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The overall goal of this project is to probe the CTL - tumor cell interaction by generating scFv probes that are able to recognize the HLA-A*0201-HER-2/$neu_{369-377}$ peptide complex. In the 12 month period covered by this report, I have successfully generated HLA-A*0201-HER-2/$neu_{369-377}$ complexes, and have isolated two scFv fragment clones that recognize this complex. In addition, I have started to analyze the expression levels of antigen processing machinery (APM) components, HLA class I antigens and β2m in several breast carcinoma cell lines. This analysis takes advantage of the availability of a wide panel of mAb to these antigens that several investigators in our laboratory, including myself, have developed and characterized. Collectively, the results we have obtained strongly support our future analysis to correlate the expression levels of APM components, HLA class I antigens, β2m and HER-2/neu with the levels of HLA-A*0201-HER-2/$neu_{369-377}$ complexes on breast carcinoma cells and lesions. The information derived from these studies is expected to contribute to our knowledge of the variables that influence the levels of HLA class I antigen-TAA derived peptide complex expression on breast carcinoma cells.

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INTRODUCTION

This Annual Summary is the first annual report of DOD Predoctoral Fellowship Award BC030039, entitled "CTL – Tumor Cell Interaction: The generation of molecular probes capable of monitoring the HLA-A*0201-HER-2/neu peptide complex". It is intended to communicate the research progress, with particular emphasis on key research accomplishments and reportable outcomes, related to this award mechanism in the 12-month period spanning from February 2004 through January 2005.

I have successfully initiated many of the project tasks outlined in the Statement of Work. In collaboration with other investigators from our laboratory, I participated in the development and characterization of a panel of monoclonal antibodies (mAb) that are specific for proteasome and immunoproteasome subunits. The availability of these mAb, along with panels of mAb that recognize other antigen processing machinery (APM) components as well as surface HLA class I antigen and β2-microglobulin (β2m), have enabled me to analyze the expression of these antigens in several breast carcinoma cell lines. In addition, I have been able to generate and purify HLA-A*0201-HER-2/$neu_{369-377}$ peptide complexes. By panning a semi-synthetic phage display scFv library with these complexes, I have successfully isolated two scFv fragment clones that react specifically with this complex.

An immediate future goal of this project will be to increase the avidity of the HLA-A*0201-HER-2/$neu_{369-377}$ peptide complex-specific scFv fragments by generating scFv tetramers. In addition, the expression levels of APM components, HLA class I antigen and β2m on other breast carcinoma cell lines and on frozen sections of breast carcinoma lesions will be analyzed. The expression of HER-2/neu will be quantitated for all cell lines and lesions. Lastly, the scFv tetramers will be utilized to analyze the levels of HLA-A*0201-HER-2/$neu_{369-377}$ peptide complexes that are expressed by the breast carcinoma cell lines and breast carcinoma lesions. The data obtained from this analysis will allow us to determine if there are correlations between the levels of APM components, HLA class I antigens, β2m and/or HER-2/neu on the levels of HLA-A*0201-HER-2/$neu_{369-377}$ peptide complex on the breast carcinoma cells.

I. Development and characterization of constitutive proteasome- and immunoproteasome-specific monoclonal antibodies (mAb). The characterization of the expression of constitutive proteasome and immunoproteasome subunits in cells, normal tissues and malignant lesions has been hampered by the lack or limited availability of constitutive proteasome and immunoproteasome subunit-specific mAb suitable for immunohistochemical or FACS staining. Delta (Y), MB1 (X) and Z are the three catalytic β subunits located in the inner rings of the constitutive proteasome, an intracellular multicatalytic complex responsible for the generation of peptides presented by HLA class I antigens to T cells. When cells are incubated with IFN-γ, delta (Y), MB1 (X) and Z are replaced by LMP2, LMP7 and LMP10, respectively, leading to the expression of immunoproteasome which generates peptides with increased affinity for HLA class I antigens. In the course of the first year of my fellowship, I participated with several other members of the Ferrone laboratory in the generation of human delta (Y), MB1 (X), Z, LMP2, LMP7 and LMP10-specific mAb secreting hybridomas from BALB/c mice immunized with peptides and recombinant fusion proteins. The mAb SY-5, SJJ-3, NB-1, SY-1, HB-2 and TO-7 were shown to be specific for delta (Y), MB1 (X) and Z, LMP2, LMP7 and LMP10, respectively, since they react specifically with the corresponding molecules when tested with a human B lymphoid LG2 cell lysate in Western blotting and with the peptide derived from each molecule in ELISA. The reactivity of the six mAb with the corresponding intracellular antigens resulted in intracellular staining when the mAb were tested with microwave-treated and saponin-permeabilized cells in indirect immunofluorescence and with formalin-fixed, paraffin-embedded tissue sections in immunohistochemical reactions. These results suggest that the constitutive proteasome and immunoproteasome subunit-specific mAb we have

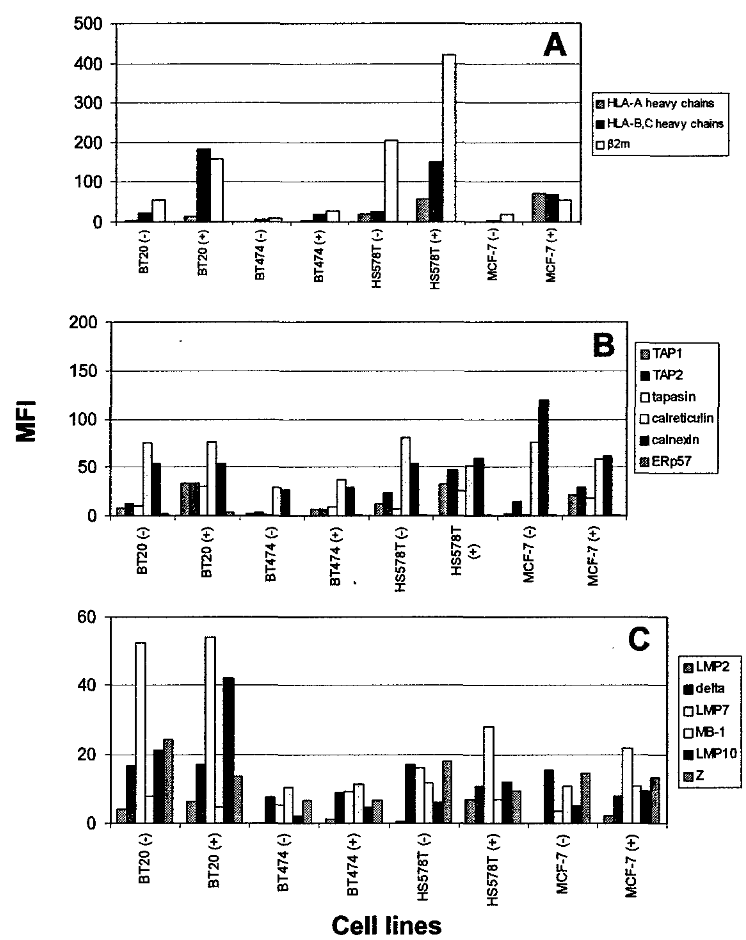

Figure 1. Heterogeneous expression of HLA class I antigens, β2m and APM components on breast carcinoma cells, and modulation of expression by IFN-γ. BT20, BT474, HS578T and MCF-7 breast carcinoma cells were either treated with 300 U/ml IFN-γ for 48 hr (+) or were untreated (-). These cells were then stained with the corresponding mAb to HLA class I antigens and β2m (Panel A), ER-resident chaperones and transporter molecules (Panel B) and proteasome and immunoproteasome components (Panel C), and were analyzed by FACS. MFI, mean fluorescence intensity.

developed are useful probes to characterize the expression of proteasome subunits in normal tissues and in pathological lesions.

II. Characterization of the level of antigen presentation machinery (APM) components, HLA class I antigen and β2m in human breast carcinoma cell lines. By taking advantage of the availability in our laboratory of a well-characterized panel of mAb specific for APM components (including the recently developed panel of mAb specific for different proteasome and immunoproteasome subunits), HLA class I antigens and β2m, we have started to analyze the expression of these antigens in a panel of 8 breast carcinoma cell lines that we have obtained. These cell lines are as follows: BT20, BT474, HS578T, MCF-7, MDA-MB-157, MDA-MB-231, SK-BR-3 and ZR75-1. In Figure 1, we have reported the progress in our analysis of four of these cell lines. Figure 1A illustrates the surface expression of HLA class I heavy chain and β2m. Figure 1B illustrates the intracellular expression of ER-resident chaperones and transporter molecules. Figure 1C illustrates the intracellular expression of proteasome and immunoproteasome subunits. In the absence of IFN-γ treatment, the basal expression levels of HLA class I, β2m and APM components were very heterogeneous among the cell lines analyzed. However, APM components such as TAP-1, tapasin (Figure 1B) and LMP10 (Figure 1C) appear to be upregulated in the presence of IFN-γ treatment (300 U/ml, 48 hr). In contrast, components such as the ER-resident chaperones, calnexin and calreticulin (Figure 1B), and the constitutive proteasome subunits, Delta and MB-1 (Figure 1C), do not appear to be upregulated in the presence of IFN-γ. These findings are generally consistent with previous findings from our laboratory in other tumor types. Of note, the upregulation of APM components such as TAP-1, tapasin and LMP10 by IFN-γ appeared to be correlated with the upregulation of HLA class I heavy chains and β2m on the surface of these cells (Figure 1A).

Table 1. CDR3 sequences and germline V gene segments from HLA-A*0201-HER-2/neu$_{369-377}$-specific scFv fragment clones

Clone	Heavy chains[a]			Light chains[a]		
	Family	Segment	CDR3[a]	Family	Segment	CDR3[a]
2.3.5	VH3	DP-13*01	AGPAGAGPWGQ	Vκ2	DPK-29*01	MQSIQLHT
2.4.38	VH3	DP-13*01	AGPAGAGPWGQ	Vλ2	DPL-19*01	NSRDSSGNHPDV

[a] Human germline VH families, human germline Vκ and Vλ gene segment families and subgroups, and CDR3 for VH, Vκ and Vλ are defined by using IMGT/V-QUEST (http://imgt.cines.fr/textes/vquest).

III. Development of HLA-A*0201-HER-2/neu$_{369-377}$ complex-specific scFv fragments. We have been successful in producing recombinant HLA-A*0201-HER-2/neu$_{369-377}$ peptide complexes. These complexes were used to isolate HLA-A*0201-HER-

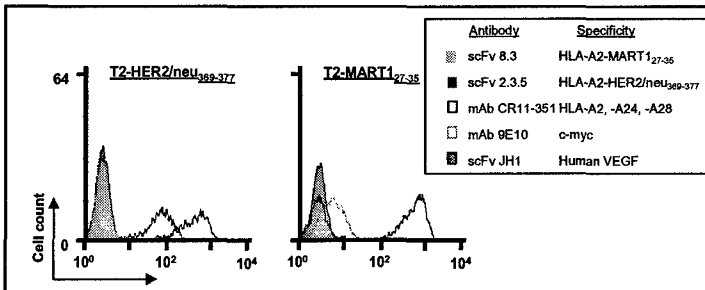

Figure 2. Reactivity of HLA-A*0201-TA peptide complex-specific scFv with peptide pulsed T2 cells. Cells were stained with HLA-A*0201-HER2/neu$_{369-377}$-specific scFv 2.3.5. Controls include the MART1$_{27-35}$ peptide-pulsed T2 cells, HLA-A*0201-MART1$_{27-35}$-specific scFv 8.3, human VEGF-specific scFv JH1, anti-HLA-A2,A24, A28 mAb CR11-351 or anti-c-myc mAb 9E10. Results were analyzed by FACS.

$2/neu_{369-377}$ peptide complex-specific scFv fragments. Briefly, we panned a semi-synthetic phage display scFv library with HLA-*0201-HER-2/$neu_{369-377}$. Seventy-two (30%) of the 240 randomly isolated clones reacted with HLA-A*0201-HER-2/$neu_{369-377}$ peptide complexes but not HLA-A*0201-$MART1_{27-35}$ peptide complexes (data not shown). Fifteen (20%) of these clones were found to react with T2 cells pulsed with HER-2/$neu_{369-377}$ peptide but not with the HLA-A*0201-binding peptide $MART1_{27-35}$ (data not shown). DNA sequence analysis of these 15 clones revealed the isolation of two unique HLA-A*0201-HER-2/$neu_{369-377}$-specific scFv (clones 2.3.5 and 2.4.38). Each scFv shares identical V_H chains but has a unique V_L chain (Table 1). Since V_H chains are predominantly involved in antibody-antigen interactions, these findings suggest that scFv 2.3.5 and 2.4.38 recognize similar determinants expressed by HLA-A*0201-HER-2/$neu_{369-377}$ peptide complexes. Therefore, studies were continued only with scFv 2.3.5. scFv 2.3.5 demonstrates specific reactivity with HER-2/$neu_{369-377}$ peptide-pulsed T2 cells, but not with T2 cells pulsed with the HLA-A*0201-binding peptide $MART1_{27-35}$ (Figure 2). Surprisingly, HLA-A*0201-HER-2/$neu_{369-377}$-peptide complex-specific scFv 2.3.5 did not bind to the HLA-A*0201, HER2/neu positive breast carcinoma cell line SK-BR-3 (data not shown). We hypothesize that this may be due to the low affinity of the isolated scFv fragment for the peptide complex.

To increase the strength of the scFv binding interaction with the HLA-A*0201-HER-$2/neu_{369-377}$ peptide complex, we are currently generating scFv tetramers to increase binding avidity. Briefly, HLA-A*0201-HER-$2_{369-377}$-specific scFv fragments will be engineered with a carboxyl-terminal BirA biotinylation tag (scFv-BirA) in two sequential PCR amplifications using the primer combinations LMB3 [Ref. 1] (5'-CAG GAA ACA GCT ATG AC-3'), located upstream of a NcoI restriction site, and scBir1 (5'-GTT CAG ACC GCC ACC TGC GTG ATG GTG ATG ATG ATG TGC GGC CCC ACG TTT GAT-3') and LMB3 and scBir2 (5'-GCG GCC GCC TAT TCG TGC CAT TCG ATT TTC TGA GCC TCG AAG ATG TC-3'), which contains a NotI restriction site, respectively (Figure 3). PCR is performed in a 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of each primer and 0.8 units of Taq polymerase (Roche) in a 50 µl reaction volume, using a GeneAmp 9700 (Applied Biosystems). An initial denaturation step will be performed for 5 min at 95°C, followed by 30 cycles of denaturation (60 sec at 94°C), annealing (60 sec at 45°C) and extension (60 sec at 72°C). Amplification will be completed with a final incubation step for 10 min at 72°C. scFv-BirA constructs will be ligated into an NcoI and Not I restriction site located on the pHEN2 vector, transformed into TG1 *E. coli* and expressed from ampicillin-resistant bacterial colonies as previously described [1-4]. Purified scFv-BirA will be biotinylated using the BirA enzyme according to the manufacturer's instructions (Avidity, Denver, CO). Tetrameric scFv complexes will be generated by linking monomeric scFv-BirA with streptavidin-PE as described [5].

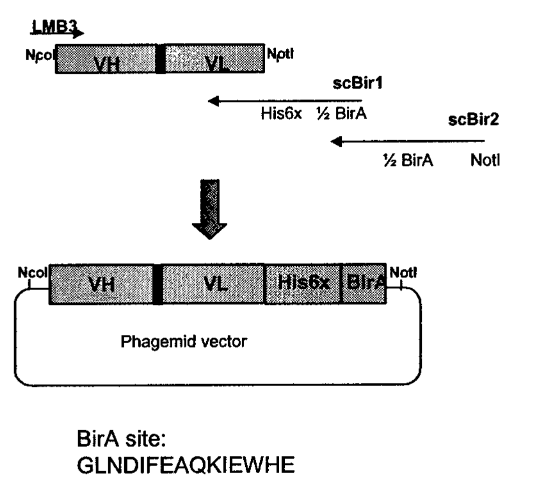

Figure 3. Insertion of the site-specific biotinylation tag BirA into scFv expression vectors to create a scFv tetramer.

KEY RESEARCH ACCOMPLISHMENTS

- Participated in the generation and characterization of a panel of mAb specific for the proteasome components delta (Y), MB1 (X) and Z and the immunoproteasome components LMP2, LMP7 and LMP10.
- Characterized the level of antigen presentation machinery (APM) components and HLA class I antigen expression in human breast carcinoma cell lines by FACS analysis, utilizing panels of mAb specific for APM components, HLA class I antigen and β2m that have been developed in the Ferrone laboratory.
- Successfully generated and purified HLA-A*0201-HER-2/$neu_{369-377}$ peptide complexes.
- Isolated two HLA-A*0201-HER-2/$neu_{369-377}$ peptide complex-specific scFv fragment clones (2.3.5 and 2.4.38) from a semi-synthetic phage-display scFv library.
- Demonstrated the ability of scFv 2.3.5 to react specifically with HER-2/$neu_{369-377}$ peptide-pulsed T2 cells.

REPORTABLE OUTCOMES

Manuscripts

1. Campoli, M., Chang, C.C. and Ferrone, S. HLA class I antigen loss, tumor immune escape and immune selection. Vaccine 20 Suppl 4:A40-45, 2002.

2. Wang, X., Campoli, M., Ko, E., Luo, and W., Ferrone, S. Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies. J Immunol Methods. 294:23-35, 2004.

3. Chang, C.C., Campoli, M., Restifo, N.P., Wang, X., and Ferrone, S. Immune selection of hot-spot β2-microglobulin gene mutations, HLA-A2 allospecificity loss and antigen process machinery component downregulation in melanoma cells derived from recurrent metastases following immunotherapy. J. Immunol. Accepted for 2005 publication.

4. Campoli, M., Chang, C.C., Oldford, S.A., Edgecombe, A.D., Drover, S., Ferrone, S. HLA antigen changes in malignant tumors of mammary epithelial origin: Molecular mechanism and clinical implications. In Immunology of breast cancer (D. Lopez and W. Wei, eds.), IOS 2005, in press.

CONCLUSIONS

In the course of the last 12 months, I have successfully initiated several of the tasks outlined in the original Statement of Work. The key accomplishments to date include (i) the development of mAb to target proteasome and immunoproteasome subunits; (ii) the analysis of expression levels of APM components, HLA class I antigen and β2m in a panel of breast carcinoma cells; (iii) the generation of HLA-A*0201-HER-2/neu$_{369-377}$ peptide complexes; and (iv) the isolation of HLA-A*0201-HER-2/neu$_{369-377}$ complex-specific scFv fragment clones. The reportable outcomes include 4 manuscripts, among which 2 have been published and an additional 2 have been accepted for publication.

In conclusion, I would like to emphasize that the research activities supported by this DOD Predoctoral Fellowship Award have served as an ideal training vehicle for a broad range of laboratory techniques, including (i) the production and screening of mAb-secreting hybridomas; (ii) the analysis of protein antigens with mAb, by utilizing Western blotting, immunohistochemistry and flow cytometry (FACS) technologies; (iii) the generation of recombinant proteins in a bacterial system by DNA manipulation with standard molecular biology methods; (iv) the generation and analysis of stable HLA-A*0201-HER-2/neu$_{369-377}$ peptide complexes; and (v) the screening of a semi-synthetic phage-display scFv library for HLA-A*0201-HER-2/neu$_{369-377}$ complex-specific scFv fragment clones.

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HLA class I antigen loss, tumor immune escape and immune selection

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Abstract

Poor clinical response rates have been observed in the majority of the T cell-based immunotherapy clinical trials conducted to date. One reason might be the presence of abnormalities in HLA class I antigen presentation in malignant lesions. An increased frequency of HLA class I abnormalities has been observed in malignant lesions from patients treated with T cell-based immunotherapy and in lesions which have recurred in patients who had experienced clinical responses following T cell-based immunotherapy. These observations are compatible with the possibility that the outgrowth of a patient's tumor reflects immune selection of tumor cells which have acquired escape mechanisms from immune recognition.

Keywords: HLA class I antigens; Malignancy; Immune selection

1. Introduction

During the last three decades, there has been an enthusiastic interest in developing and applying immunotherapy for the treatment of malignant diseases, due to: (i) the limited efficacy of chemotherapy; (ii) the major progress made in the identification of tumor antigens (TA) and corresponding probes [1]; and (iii) the substantial increase in our understanding of the molecular steps leading to an immune response. Active specific T cell-based immunotherapy has been emphasized in part because of the disappointing results of the antibody-based trials conducted in the early 1980s [2] and in part because of the general belief that T cells play a major role in tumor growth control [3]. Taking advantage of the many TA identified in malignant cells and of the multiple ways to modulate an immune response, an increasing number of active specific immunotherapeutic strategies have been developed [4]. Because of the emphasis on translational research, those strategies, yielding promising results in animal model systems, have been utilized to implement phases I and II clinical trials [4].

The results of these investigations have highlighted two challenges facing tumor immunologists and clinical oncologists. The first is the selection of the most effective immunotherapeutic strategy, since the various available strategies have not been compared in a systemic way and this information is not likely to become available in the near future given the prohibitive costs of clinical trials. The second is the understanding of why a TA-specific immune response, which can be detected in a variable percentage of patients, is not paralleled by a clinical response in the majority of immunized patients [4]. It is our contention that this discrepancy is likely to reflect immune escape mechanisms due to the frequent antigenic changes which occur in human malignant cells because of their genetic instability [5] and usually long duration of the disease, providing them with immune escape mechanisms. In this paper, we will discuss the defects of the HLA class I antigen processing machinery frequently found in malignant cells and their clinical significance, since this machinery plays a crucial role in the interaction of tumor cells with cytotoxic T lymphocytes (CTL). As schematically shown in Fig. 1, TA derived peptides are generated by proteosomal cleavage and transported into the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP). Subsequently, tapasin plays a role in the selection of peptides to be loaded onto the HLA class I heavy chain–β_2-microglobulin (β_2m) complex. Properly folded HLA class I antigen–TA derived peptide complexes are then transported to the cell surface and recognized by CTL through the T cell receptor. Because of space limitations we will succinctly discuss these topics; for a detailed discussion we refer the interested reader to our recent papers [6,7].

2. HLA class I antigen expression in malignant lesions

Analysis of HLA class I antigen expression by malignant cells of nonlymphoid origin was initially restricted

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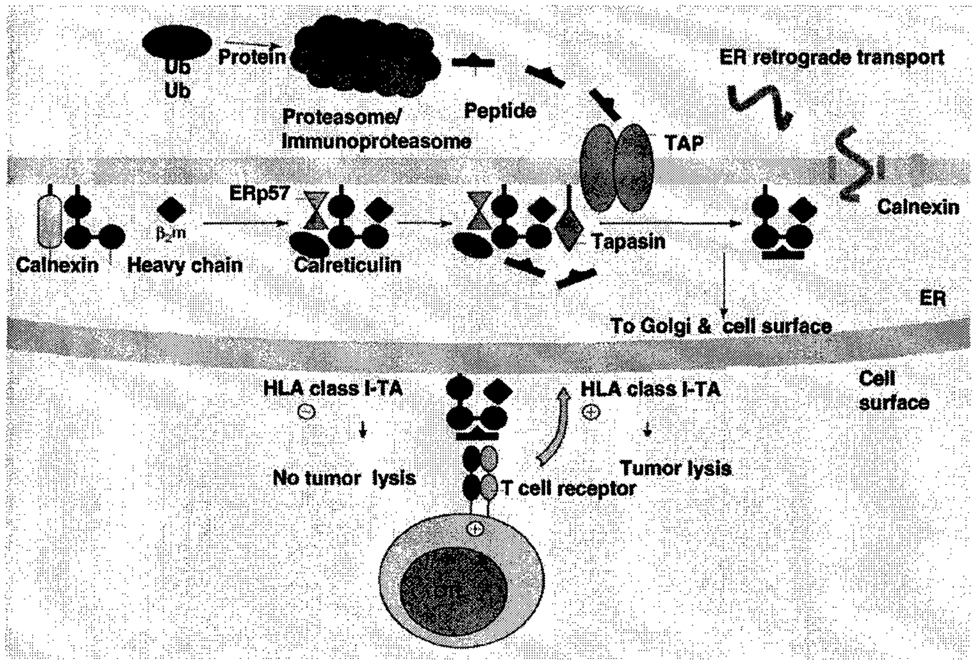

Fig. 1. HLA class I antigen processing and presentation. Protein antigens are marked for ubiquitination within the cytosol and subsequently degraded by the proteasome. Peptides are then transported into the ER through TAP. HLA class I antigens are translated and transported into the ER. Within the ER, the HLA class I heavy chain associates with several chaperone proteins (BiP, calnexin, calreticulin and ERp57) and with β_2m. These chaperone proteins assist in the proper folding of the HLA class I molecule. The HLA class I–β_2m complex then associates with tapasin. Tapasin brings the dimeric complex into association with TAP and ensures proper peptide loading onto the HLA class I–β_2m complex. The trimeric HLA class I–β_2m complex-peptide complex is then transported to the plasma membrane.

to cell lines in long-term culture, since the limited specificity of the conventional allo- and xeno-antisera did not allow one to apply immunohistochemical (IHC) techniques to analyze surgical lesions. This limitation was overcome by the development of anti-HLA class I monoclonal antibodies (mAb). Based on the characteristics of their specificity, distinct phases can be identified in the studies which have analyzed HLA class I antigen expression in malignant lesions.

In the 1980s, IHC staining of a large number of malignant lesions with mAb to monomorphic determinants of HLA class I antigens identified changes in the expression of these antigens in all the types of tumors analyzed [6]. With the exception of liver carcinoma [8,9] in all the tumors analyzed, malignant transformation of cells is associated with HLA class I antigen loss or downregulation. In liver, normal hepatocytes, which do not express or express very low levels of HLA class I antigens [8,9], may acquire these antigens when they undergo malignant transformation. As reviewed in [6], the frequency of HLA class I antigen loss or downregulation has been found to be between about 15% in primary melanoma lesions and 50% in primary prostate carcinoma lesions. The reason for these differences is not known. They are likely to reflect the time length between onset of tumor and diagnosis, since a long interval gives tumor cells more chances to mutate in the genes involved in HLA class I antigen expression and allows mutated cells to over-grow cells without abnormalities in their HLA class I phenotype in the presence of T cell selective pressure, as will be discussed later. This reason may also account for the low frequency of HLA class I antigen abnormalities in various types of leukemia [10].

In subsequent years, mAb to HLA class I allospecificities have been utilized to phenotype malignant lesions. Unfortunately, the large majority of the available mAb to HLA class I allospecificities were found not to work in IHC reactions. As a result, the expression of only a limited number of HLA class I allospecificities has been investigated in malignant lesions. Nevertheless, these studies have identified different types of selective HLA class I allospecificity loss or downregulation. They include loss or downregulation of one HLA class I allospecificity, loss of the allospecificities encoded in one haplotype and downregulation of the gene products of one locus (Fig. 2). From a practical viewpoint, it is noteworthy that these selective losses cannot be detected by staining malignant lesions with mAb to framework determinants of HLA class I antigens. On the other hand, at least some of these selective losses can be identified using mAb which recognize determinants restricted to the gene products of one HLA class I locus. Therefore, the latter probes may alleviate the restrictions imposed on these studies by the lack of mAb to HLA class I allospecificities suitable for IHC studies. The frequency of selective HLA class I allospecificity loss and downregulation has been found to be in the range of 15–51% depending on the type of

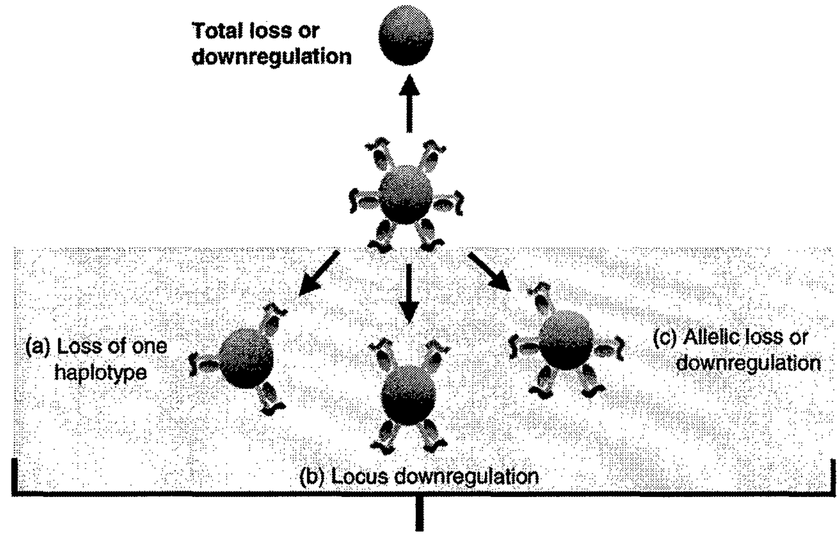

Fig. 2. Phenotypes of altered HLA class I antigen expression identified in human malignancies. A tumor with a normal HLA class I phenotype can give rise to several altered phenotypes. Those identified in human tumors and characterized in tumor cell lines include HLA class I total loss or downregulation and selective HLA class I loss or downregulation. The latter includes: (a) loss of a haplotype; (b) locus specific downregulation; (c) loss or downregulation of an allele; or multiple defects may be present in a tumor cell resulting in a complex phenotype.

malignancy [11]. It is higher in melanoma, cervical carcinoma and prostate carcinoma than in head and neck squamous cell carcinoma, breast carcinoma, lung carcinoma and colon carcinoma.

In recent years, the expression of components of the antigen processing machinery has been analyzed. Because of the lack of mAb with appropriate characteristics, initially only the expression of the proteasome subunits LMP2 and LMP7 and of TAP1 has been investigated in malignant lesions [12]. The frequency of TAP1 downregulation varies significantly in different tumor types, ranging from 14% in primary colorectal carcinoma to 49% in primary human papilloma virus 16 (+) cervical carcinomas [12]. The frequency of LMP2 and LMP7 downregulation has primarily been analyzed in melanoma where its frequency ranges from 37 to 55% in primary lesions and 19 to 53% in metastatic lesions, respectively. Only during the last year has tapasin expression been analyzed in a few types of tumors. Tapasin loss or downregulation has been found with high frequency in various types of malignancies (unpublished data). It is likely that these studies will be extended to the other components of the antigen processing machinery as mAb with the appropriate characteristics become available.

It is noteworthy that especially in malignant cells isolated from patients with advanced disease the presence of multiple defects affecting different components of the antigen processing machinery and HLA class I subunits appears to be the rule more than the exception. An example is shown in Fig. 2, tapasin is not detectable in the melanoma cell line SK-MEL-33 which does not express HLA class I antigens because a single base deletion in the β_2m gene generates a premature stop codon which prevents its translation [13].

The investigations performed thus far have only analyzed the expression of HLA class I subunits and of select components of the antigen processing machinery in malignant lesions. Although, conclusive when proteins are not detected, these studies do not provide any information about the function of the proteins expressed and about the functional implications of downregulation of one or more components of the HLA class I antigen processing machinery. Therefore, expression of HLA class I proteins cannot be taken as evidence that peptides are generated from TA and presented by HLA class I allospecificities to CTL. This possibility is supported by the recent finding that melanoma cells without detectable defects in the HLA class I antigen processing machinery and in the melanoma-associated antigen MART-1 expression are not recognized by HLA-A2-restricted, MART-1-specific CTL, since LMP2 and LMP7 expression in the immunoproteosome inhibits MART-1-derived peptide presentation to CTL [14]. Furthermore, it is likely that defects in tapasin expression and/or function which appear to be frequent in malignant cells will change the repertoire of peptides presented by HLA class I antigens to CTL. These possibilities provide a mechanism for resistance to CTL-mediated lysis of tumor cells without detectable defects in HLA class I antigen expression and stress the need to develop probes to monitor the expression of HLA class I antigen–TA derived peptide complexes on tumor cells. In this regard, recently published studies [15,16] and our own results indicate that phage-display antibody libraries may be a useful source for these types of probes.

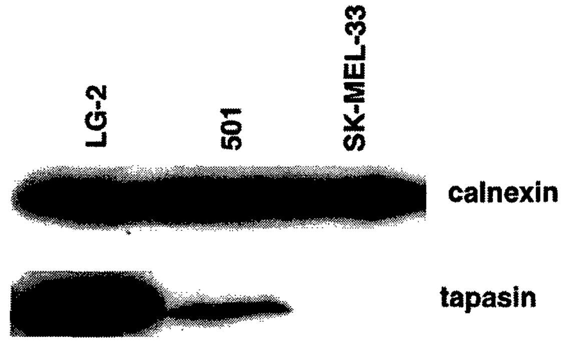

Fig. 3. Multiple defects in HLA class I antigen presentation in melanoma cell line SK-MEL-33. Melanoma cell line SK-MEL-33, which does not express HLA class I antigens because of a deletion in the β_2m gene, does not express tapasin as shown by the lack of reactivity of a cell lysate with the anti-tapasin mAb TO-3 in Western blotting. The human lymphoid cell line LG-2 and the human melanoma cell line 501 are used as positive controls.

3. Association of HLA class I antigen changes in malignant lesions with histopathological and clinical parameters

HLA class I antigen defects are likely to play a role in the clinical course of malignant disease. In many types of tumors, the frequency of HLA class I antigen defects is increased in metastatic lesions when compared to primary lesions. Furthermore, in melanoma and cervical carcinoma, the frequency of HLA class I abnormalities in primary lesions is higher than in premalignant lesions and there is an association with histopathologic progression [6]. In the majority of the carcinoma lesions analyzed to date, HLA class I antigen downregulation is significantly associated with poor histological differentiation, abnormal DNA content and advanced clinical stage (tumor grading), each suggestive of more aggressive tumors [6]. Possible explanations for the observation that abnormalities in HLA class I antigen expression are associated with later stage and less differentiated tumors include immune selection of tumors cells with abnormalities in HLA class I antigen expression, accumulation of mutations by tumor cells or technical aspects of analysis. Lastly, in various types of malignancies, HLA class I antigen downregulation is associated with a reduction in disease free interval and survival (Fig. 3) [6]. The only exception is represented by uveal melanoma, where HLA class I antigen downregulation is associated with a decrease in metastases and improved survival [17].

4. Molecular defects underlying HLA class I antigen abnormalities

Characterization of cell lines originated from malignant lesions with HLA class I abnormalities has shown that distinct molecular mechanisms underlie the abnormal HLA class I phenotypes of tumor cells [18]. Total HLA class I antigen loss is caused by defects in β_2m which is required for expression of the HLA class I trimolecular complex on the cell membrane. The β_2m defects result from two events: loss of one copy of the β_2m gene and mutations in the other copy which inhibits its transcription in a few cases and its translation in most cases. From a practical viewpoint, the latter finding emphasizes the need to test malignant lesions with antibodies in IHC assays and not to utilize in situ hybridization to assess β_2m transcript expression. Selective HLA class I antigen loss is caused by loss of the gene(s) encoding the lost HLA class I heavy chain(s) or by mutations which inhibit their transcription or translation. As in the case of the β_2m gene, the mutations found in HLA class I heavy chains range from large deletions to single base deletions [18]. The mutations appear to occur randomly.

In contrast to HLA class I antigen loss, HLA class I antigen downregulation is usually caused by defects in the

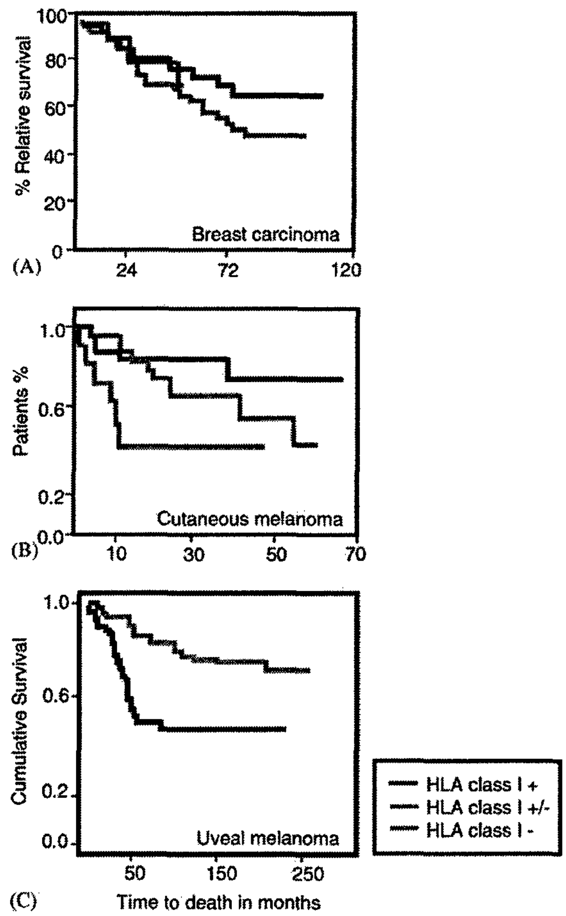

Fig. 4. Association between HLA class I antigen expression and patients' survival in breast carcinoma and melanoma. Reduced survival is seen in patients with (A) breast carcinoma and (B) cutaneous melanoma with abnormal HLA class I antigen expression, suggesting a role of HLA class I antigen restricted, TA-specific CTL in the clinical course of the disease. (C) In contrast, in patients with uveal melanoma, HLA class I antigen downregulation is associated with an increase in survival, suggesting a role for NK cells influencing in the clinical course of the disease.

regulatory mechanisms which control HLA class I antigen expression and/or abnormalities in one of the components of the antigen processing machinery [12]. It is noteworthy that in most of these cases, HLA class I antigen expression can be restored by cytokines. Therefore, these patients, at variance with those with structural defects in HLA class I antigen-encoding genes, are likely to benefit by combining T cell-based immunotherapy with administration of cytokines.

5. Role of selective pressure in the generation of lesions with HLA class I defects

The first event in the generation of lesions with HLA class I defects is represented by a mutation in a gene(s) involved in HLA class I antigen expression. The available information suggests that these mutations occur randomly because of tumor cell genetic instability [5]. In vitro experiments and experiments in animal model systems suggest that tumor cells with an HLA class I antigen defect outgrow malignant cells with a normal HLA class I antigen phenotype when exposed to CTL which utilize the defective HLA class I allospecificity to recognize tumor cells (Fig. 4). On the other hand, no significant changes in the HLA class I antigen phenotype are detected when the tumor cell population is grown in an immunologically naïve environment.

The clinical counterpart of these findings is represented by the increased frequency of HLA class I losses in malignant lesions in patients treated with T cell-based immunotherapy [19]. Furthermore, loss of HLA class I antigens has been frequently found in lesions which have recurred in patients who had experienced clinical responses following T cell-based immunotherapy [19,20]. These findings have three implications. First, the presence of malignant lesions with HLA class I antigen defects in patients argues in favor of the possibility that the patients' immune system has mounted an immune response against his own tumor and that this immune response has favored the growth of the tumor cell population which has acquired resistance because of an HLA class I abnormality. If this interpretation is correct, the outgrowth of tumors in patients is likely to reflect escape of tumor cells from immune recognition more than dormancy or ignorance of the patient's immune system. This possibility is supported by the disease progression frequently observed in patients with malignancy in spite of the development and persistence of a TA-specific CTL response [4]. Second, the presence of multiple defects affecting HLA class I antigen expression at different levels in tumor cells is compatible with the possibility that a patient's immune system targets a different HLA class I antigen–TA derived peptide complex, when the tumor cell population develops an escape mechanism from the ongoing immune response (Fig. 5). If this interpretation is correct, tumor growth is likely to occur when the multiple escape mechanisms developed by tumor cells have exhausted the repertoire of TA-specific immune responses the patient can develop. Third, given the high rate of mutations in tumor cells, T cell-based immunotherapy, even when successful, is not likely to be able to eradicate a patient's malignancy, since the eventual outcome is likely to be the outgrowth of tumor cells which have developed multiple escape mechanisms. If this interpretation is correct, multiple TA and multiple immune effector mechanisms should

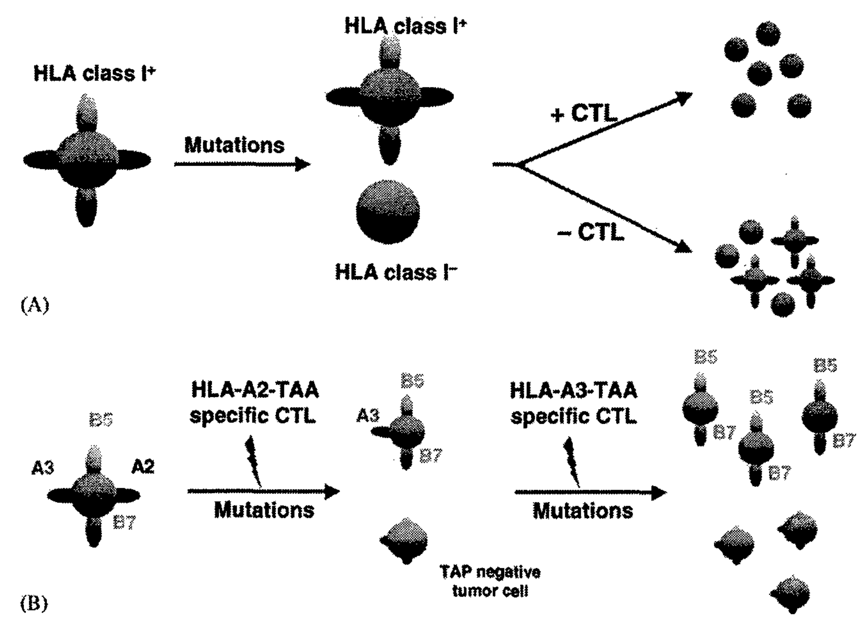

Fig. 5. Role of immune selection in the generation of malignant lesions with HLA class I defects. (A) Tumor cells with an HLA class I antigen defect outgrow malignant cells with a normal HLA class I antigen phenotype when exposed to CTL which utilize the defective HLA class I allospecificity to recognize tumor cells. (B) Multiple defects affecting HLA class I antigen expression at different levels in tumor cells may reflect the adaptation of a patient's immune system to target different HLA class I antigen-TA derived peptide complexes in the control of tumor growth.

be utilized when targeting a patient's tumor. In addition, the concomitant targeting by immune therapy of normal cells, which play a role in tumor growth, may alleviate the problems caused by the genetic instability of malignant cells.

6. Conclusion

Our understanding of how the immune system interacts with tumor cells has greatly improved over the last decade. Tumor cell genetic instability gives them the ability to alter their antigenic profile and avoid immune destruction. This ability remains an important obstacle for the treatment of human malignancies through T cell-based immunotherapy. Understanding the molecular mechanisms behind tumor cell escape from immune destruction will provide valuable insight in designing effective T cell-based immunotherapeutic strategies.

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Research paper

Enhancement of scFv fragment reactivity with target antigens in binding assays following mixing with anti-tag monoclonal antibodies

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Abstract

The phage display Ab library technology has been found to be a useful method to isolate antigen-specific Ab fragments, since the repertoire of antibody specificities is broad and since it bypasses the need of immunization. However, when screening clones isolated from a phage display Ab library, the yield of isolating antigen-specific Ab fragments is low and the rate of false negative results is high. This limitation reflects the low affinity/avidity of Ab fragments and/or the low density of the target antigen. To facilitate the isolation of Ab fragments with a broad range of affinities to antigens of interest from phage display Ab libraries, we have developed a simple method to increase the sensitivity of binding assays to detect the reactivity of single-chain fragments of antibody variable regions (scFv) with target antigens. This method involves the mixing of scFv fragments, expressing a c-myc epitope tag, with anti-tag mAb 9E10 prior to their use in binding assays in order to form stable dimeric Ab fragment–anti-tag mAb complexes. The increase in the reactivity of scFv fragments with the corresponding antigen is observed over a broad range of scFv fragment (6–800 μg/ml) and mAb 9E10 (0.5–30 μg/ml) concentrations, thereby facilitating the testing of scFv fragment preparations with unknown scFv fragment concentrations. Use of this method in binding assays resulted in a twofold increase in the reactivity of low-affinity purified scFv fragments with the corresponding antigen. Moreover, application of this method to screen clones isolated from phage display scFv libraries resulted in a reproducible increase in both the yield of antigen-specific scFv clones and the titer of scFv fragment preparations by a factor of 5 and 2- to 32-fold, respectively. Lastly, this method can be applied in both ELISA and flow cytometry and is independent of the characteristics of the antigen (i.e. whole cells, carbohydrates and purified protein) and/or of the library (synthetic scFv Library (#1), a large semi-synthetic phage display scFv library and the human synthetic VH+VL scFv library (Griffin.1 library)) used. Therefore, the method we have

Abbreviations: Ab, antibody; CDR, complementarity determining region; ELISA, enzyme-linked immunosorbent assay; HMW-MAA, high molecular weight-melanoma-associated antigen; mAb, monoclonal antibody; scFv, single-chain fragment of antibody variable region; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TMB, 3,3',5,5'-tetramethylbenzidine.

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described represents a sensitive, simple and reproducible technique that will facilitate the isolation and use of scFv fragments.

Keywords: Phage library; scFv

1. Introduction

In recent years, the phage display Ab library technology, constructed either with naïve genes with in vitro rearranged complementarity determining region 3 (CDR3) or genes from immunized individuals, has become a very popular technique (Griffiths and Duncan, 1998, Hoogenboom et al., 1998). This is due in part to the ability of phage display technology to bypass the need of immunization to generate Ab of desired specificity. As a result, the isolation of Ab fragments with the desired specificity is not dependent on the immunogenicity of the corresponding antigen. Moreover, the range of specificity present in synthetic phage display Ab libraries is greater than that found in a panel of hybridomas generated from an immunized mouse (Hoogenboom et al., 1998). These advantages have facilitated the development of Ab fragments to a number of unique antigens including small molecular compounds (haptens) (Hoogenboom and Winter, 1992), molecular complexes (Chames et al., 2000), unstable compounds (Kjaer et al., 1998) and cell surface proteins (Desai et al., 1998). The smaller size of Ab fragments facilitates their modification at the DNA level in order to increase their binding affinity (Griffiths and Duncan, 1998, Hoogenboom et al., 1998), or to allow for conjugation to effector molecules (e.g., peptide, toxins) (King et al., 1998) or to each other (e.g., to generate bispecific Ab) (Hayden et al., 1994). In addition, Ab fragments have greater and faster tissue penetration, low kidney uptake and rapid blood clearance (Wels et al., 1992, Mao et al., 1999, Mayer et al., 2000, Power et al., 2001). These characteristics make them useful carriers of radioisotopes and drugs in vivo (Wels et al., 1992, Mao et al., 1999, Mayer et al., 2000, Power et al., 2001).

While the advantages of phage display Ab libraries have given rise to many useful reagents, the use of Ab fragments has some limitations. In this regard, Ab fragments often have a low affinity and avidity for the target antigen compared to conventional bivalent monoclonal antibodies (mAb) (Pini and Bracci, 2000). DNA sequences can be modified to increase both affinity and avidity once Ab fragments have been isolated and characterized (Hudson and Kortt, 1999, Pini and Bracci, 2000, Power and Hudson, 2000). However, the low affinity and avidity of Ab fragments have a negative impact on the identification of antigen-specific Ab fragments when screening clones isolated from a phage display Ab library after panning with the target antigen. Consequently, the yield of isolating antigen-specific Ab fragments is low and the rate of false negative results is high either because of their low affinity/avidity and/or because of the low density of the target antigen. To facilitate the isolation of Ab fragments with a broad range of affinities to antigens of interest from phage display Ab libraries, we have developed a simple, sensitive and reproducible method to increase the sensitivity of binding assays to detect the reactivity of scFv fragments with target antigens. The aim of this paper is to describe this method and its application in the screening and characterization of clones isolated from phage display scFv libraries by panning with various types of antigens.

2. Materials and methods

2.1. Cell lines and antigens

Cultured human melanoma cells Colo 38, FO-1 and SK-MEL-28 and lymphoblastoid cells LG-2 and T2 were maintained in RPMI 1640 medium (Tissue Culture Media Facility, Roswell Park Cancer Institute, Buffalo, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN, Costa Mesa, CA) or with 10% serum plus (Hazelton Biologics, Lenexa, KS) in a 5% CO_2 atmosphere at 37 °C.

Preparation of cell lysates and isolation of antigens from cell lysates by binding to a solid phase tube coated with a mouse mAb were performed as previously described (Desai et al., 1998). Purified melanoma-associated antigen (MAA) p97 was provided by Drs. K.E. Hellstrom and I. Hellstrom (Pacific Northwest Research Institute, Seattle, WA). $\alpha_{1,6}$ dextran N279 was provided by the late Dr. E.A. Kabat (Columbia University, New York, NY). Recombinant biotinylated HLA-A*0201-MART1$_{27-35}$ and HLA-A*0201-HER-2/neu$_{369-377}$ peptide complexes were purchased from Beckman Coulter Immunomics (Fullerton, CA).

2.2. mAb, polyclonal antibodies and scFv fragments

The mouse anti-human high molecular weight-melanoma-associated antigen (HMW-MAA) mAb TP61.5 (Chen et al., 1991), anti-HLA-A2, A24, A28 mAb CR11-351 (Russo et al., 1983) and anti-c-myc mAb 9E10 (Evan et al., 1985) were developed and characterized as described. mAb were purified from BALB/c/SCID mouse ascites by sequential ammonium sulfate and caprylic acid precipitation (Temponi et al., 1989). The purity and activity of the mAb preparations were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA), respectively. Mouse mAb were biotinylated using succinimidyl-6-biotinamido-hexanoate (NHS-LC-Biotin; Pierce, Rockford, IL) according to the manufacturer's instructions.

Phycoerythrin (PE)-conjugated goat anti-mouse Fc F(ab')$_2$ fragments and horseradish peroxidase (HRP)-conjugated sheep anti-M13 antibodies were purchased from DAKO (Carpinteria, CA) and Amersham Pharmacia (Piscataway, NJ), respectively. HRP-conjugated goat anti-mouse Fc antibodies and purified rabbit anti-mouse IgG (H+L) antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Human anti-HMW-MAA scFv #61 (Desai et al., 1998) and scFv #28 (Noronha et al., 1998) were isolated from synthetic scFv Library (#1) (Nissim et al., 1994) as described. The C and F series of human anti-MAA scFv fragments were isolated from the large semi-synthetic phage display scFv library (De Kruif et al., 1995) by panning on human melanoma cells Colo 38 and FO-1, respectively (unpublished results). Anti-HLA-A*0201-MART1$_{27-35}$ scFv 8.3 and anti-HLA-A*0201-HER-2/neu$_{369-377}$ scFv 2.3.5 were isolated from the human synthetic VH+VL scFv library (Griffin.1 library) (Griffiths et al., 1994) by panning with biotinylated HLA-A*0201-MART1$_{27-35}$ and HLA-A*0201-HER-2/neu$_{369-377}$ peptide complexes, respectively (Campoli et al., manuscript in preparation).

2.3. Synthetic peptides

The synthetic peptides representing residues 27–35 (AAGIGILTV) of MART1 (MART1$_{27-35}$) and residues 369–377 (KIFGSLAFL) of HER-2/neu (HER-2/neu$_{369-377}$) were purchased from the Molecular Genetics Instrumentation Facility at the University of Georgia Research Service (Atlanta, GA).

2.4. Selection of antigen-reactive phage from phage display antibody libraries

Panning of the synthetic scFv library (#1) was performed in immunotubes (NUNC MaxiSorp; NUNC, Roskilde, Denmark) coated with HMW-MAA captured from a Colo 38 melanoma cell lysate by mouse anti-HMW-MAA mAb TP61.5, as described (Desai et al., 1998). Panning of the synthetic scFv Library (#1) on human melanoma cells SK-MEL-28, and panning of the large semi-synthetic phage display scFv library on human melanoma cells Colo 38 and FO-1, was performed as described (Noronha et al., 1998).

2.5. Preparation of phage displayed, soluble and purified scFv fragments

Soluble scFv fragments in bacterial supernatants (SNT) and soluble scFv fragments in periplasmic preparations (PP) were produced from individual ampicillin-resistant colonies, as previously described (Skerra and Pluckthun, 1988, Marks et al., 1991, Schier et al., 1996). ScFv fragments were purified using Ni-affinity chromatography (Evan et al., 1985) following the manufacturer's instructions. Immobilized metal affinity chromatography (IMAC) was performed with beaded agarose iminodiacetic chelating resin (Sigma, St. Louis, MO). IMAC columns were charged by washing with 5 column volumes of 0.1 M $NiSO_4$ and 5 column volumes of binding buffer (BB) (0.5 M NaCl, 5 mM imidazole, 20 mM Tris–HCl, pH 8.0) (Canaan-Haden et al., 1995). Subsequently, periplasmic scFv preparations previously dialyzed against BB were loaded onto activated IMAC columns. Columns were washed with 10 column volumes of BB containing 50 mM imidazole and scFv fragments were eluted from IMAC columns in strip buffer (BB+0.05 M EDTA). Fractions containing scFv fragments identified by SDS-PAGE analysis were pooled and dialyzed against PBS. The purity of scFv fragment preparations as determined by SDS-PAGE was approximately 85–90%.

2.6. Peptide loading of T2 cells

T2 cells were loaded with peptides, as described (Valmori et al., 1998). Briefly, T2 cells (4×10^6/ml) were washed twice with serum-free RPMI 1640 medium and then incubated at room temperature (RT) overnight with 100 μM of synthetic peptide $MART1_{27-35}$ or of $HER2/neu_{369-377}$. Subsequently, cells were washed twice with 2%BSA-PBS and used in flow cytometric assays.

2.7. Preparation of ELISA plates with purified antigens and with cells

mAb CR11-351 (1 μg/well), $\alpha_{1,6}$ dextran N279 (10 μg/well) or recombinant streptavidin (Roche Diagnostics, Mannheim, Germany) (1 μg/well) were added to 96-well, flexible, U-bottom Costar 2797 microtiter plates (Corning Costar, Cambridge, MA) in 50 mM $NaHCO_3$, pH 9.6. Following an overnight incubation at 4 °C, plates were blocked with 2% BSA-PBS for 2 h at RT. Streptavidin coated plates were then incubated with biotinylated HLA class I antigen–peptide complexes (20 ng/well) for 2 h at RT. For cell ELISA, cultured cells (2×10^5/50 μl cell culture medium/well) were transferred to Costar 3595 microtiter plates (Corning Costar). Plates were then used in ELISA.

2.8. Binding assays

Unless otherwise noted, the modified binding assay to detect the reactivity of scFv fragments with target antigens was performed by first mixing 50 μl of a scFv fragment preparation with an equal volume of a mAb 9E10 preparation (0.5–30 μg/ml) at RT. Subsequently, scFv fragment–mAb 9E10 mixtures were transferred to antigen- or cell-containing wells and incubation was continued for 2 h for ELISA and for 1 h for flow cytometry. In nonmodified binding assays, scFv fragments are mixed directly in antigen- or cell-containing wells with an equal volume of 2% BSA-PBS for 2 h for ELISA or for 30 min for flow cytometry. Subsequently, 50 μl of a mAb 9E10 preparation (0.5–30 μg/ml) is added to each well. The mixture is incubated for 1 h for ELISA and for 30 min for flow cytometry. All incubations were carried out at RT for antigen-ELISA and at 4 °C for flow cytometry and cell ELISA.

For ELISA with scFv fragment SNT and PP, wells were washed five times with 0.05% Tween-20/PBS and three times with PBS between each incubation. For cell ELISA with scFv fragment SNT and PP, wells were washed three times with 0.5% BSA-PBS and once with PBS between each incubation. For purified scFv fragment ELISA, wells were washed six times with 0.05% Tween-20/PBS and five times with PBS between each incubation. Binding of scFv fragments was detected with HRP-conjugated streptavidin (SA-HRP; Pierce) when biotinylated mAb 9E10 (b-9E10) was used and with HRP-conjugated goat anti-mouse IgG antibodies (Fc-specific) when unconjugated mAb 9E10 was used. Results are expressed as optical density (OD) absorbance at 490 nm when using *ortho*-phenylenediamine (Sigma)–H_2O_2 as substrate or at 450 nm when using 3,3′,5,5′-tetramethylbenzidine (TMB) as substrate (KPL, Gaithersburg, MD) on an ELISA plate reader (Finstruments Model 348, Helsinki, Finland). Binding of purified scFv fragments in flow cytometric assays was detected using an optimal amount of recombinant PE-conjugated streptavidin. After washing off excess mAb, cells were fixed with 0.5% paraformaldehyde (Sigma) in PBS and analyzed on FACScan (Becton-Dickinson, San Jose, CA) with CellQuest™ software (Becton-

Dickinson). Results are expressed as mean fluorescence intensity (MFI).

3. Results

3.1. Increased reactivity of purified scFv fragments in binding assays following mixing with anti-tag mAb 9E10

To determine if the generation of scFv fragment–anti-tag mAb complexes could increase the sensitivity of binding assays to assess the reactivity of scFv fragments with the corresponding antigens, we tested the effect of mixing purified scFv fragments with anti-c-myc mAb 9E10 on their reactivity with the corresponding antigen. mAb 9E10 was used at concentrations ranging from 100 to 1 μg/ml. Purified scFv 8.3 was used at concentrations ranging from 800 to 0.4 μg/ml. The two antibody preparations were mixed for 1 h at RT or for 24 h at 4 °C. At all antibody concentrations tested, mixing scFv 8.3 with mAb 9E10 for 1 h or 24 h increased its reactivity with HLA-A*0201-MART1$_{27-35}$ complexes. The optimal concentration was 20 μg/ml for both mAb 9E10 and scFv 8.3. Fig. 1 shows the mean value±standard deviation of the results generated from three independent experiments. As indicated, the reactivity of purified scFv 8.3 with the HLA-A*0201-MART1$_{27-35}$ complex could not be detected. In contrast, upon mixing mAb 9E10 with scFv 8.3 for 1 or 24 h, the reactivity of scFv 8.3 increased 15-fold, suggesting that the formation of scFv 8.3–mAb 9E10 complexes increases the avidity of scFv 8.3 to the HLA-A*0201-MART1$_{27-35}$ complex. Mixing scFv 8.3 with mAb b-9E10 for 1 h also resulted in a log-fold increase in the reactivity of scFv 8.3 with MART1$_{27-35}$ pulsed T2 cells (Fig. 2A). ScFv 8.3 that was not mixed with mAb 9E10 did not demonstrate any reactivity with the MART1$_{27-35}$ pulsed T2 cells. These findings were reproducible over three independent experiments

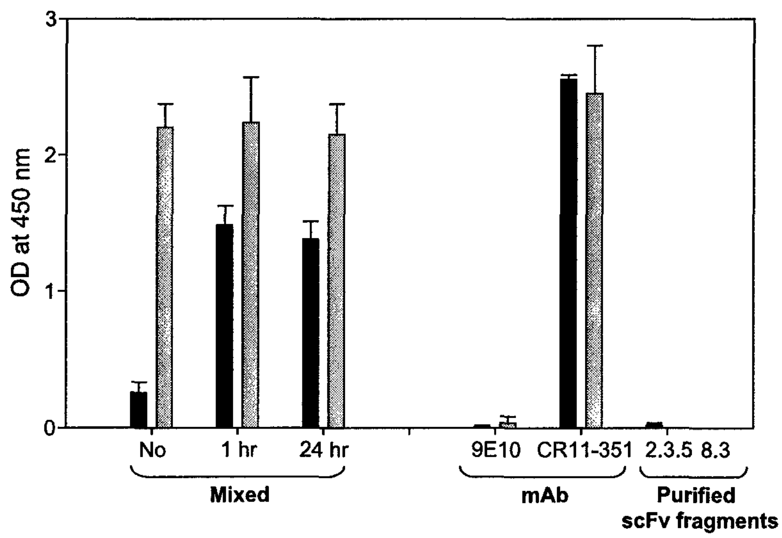
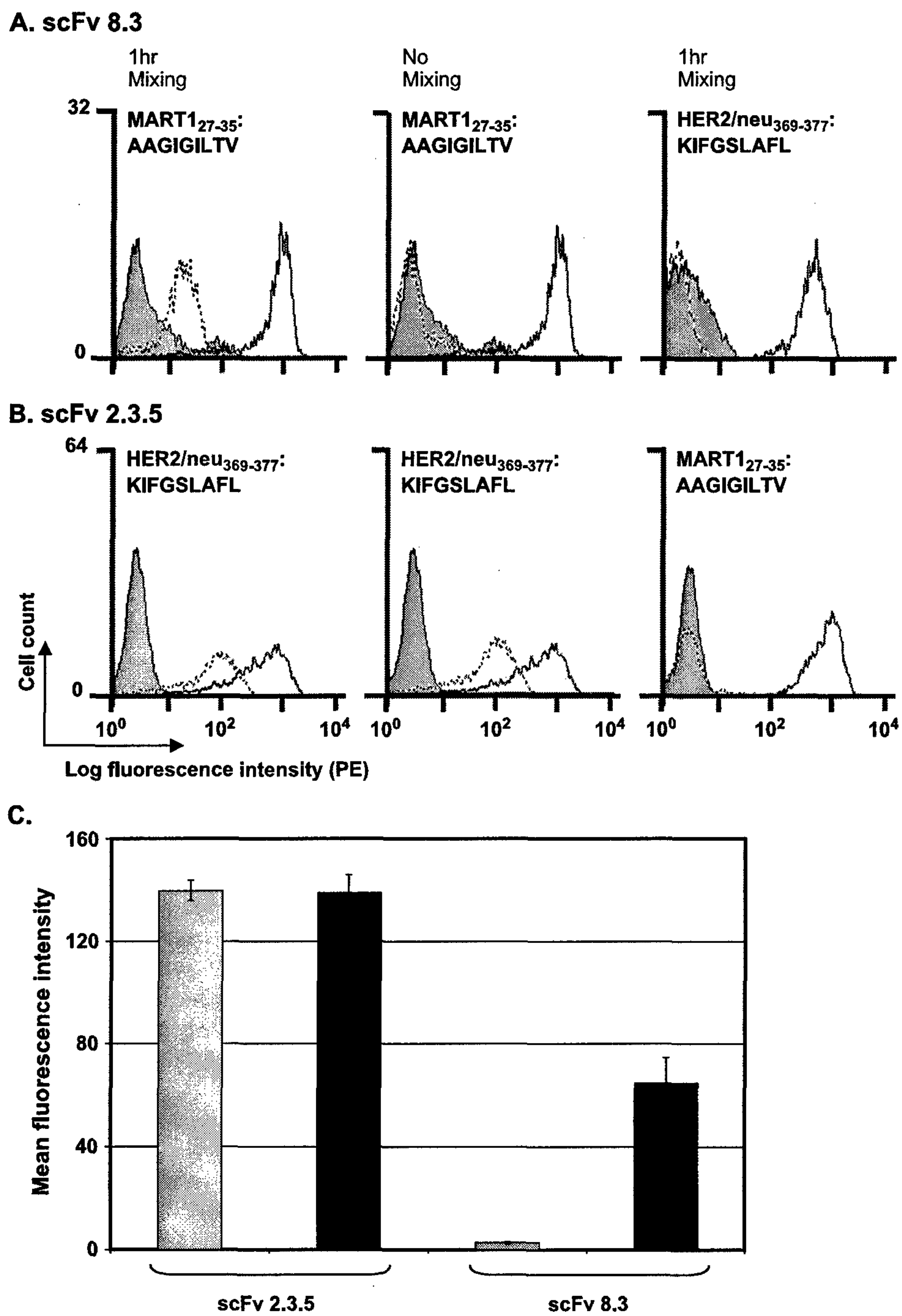


Fig. 1. Increased reactivity of purified scFv fragments in ELISA following mixing with anti-tag mAb 9E10. Biotinylated HLA-A*0201-MART1$_{27-35}$ (■) and HLA-A*0201-HER-2/neu$_{369-377}$ (▒) complexes (20 ng/well) were immobilized on a 96-well microtiter plate. Bound HLA-A*0201-MART1$_{27-35}$ and HLA-A*0201-HER-2/neu$_{369-377}$ complexes were incubated with 50 μl (20 μg/ml) of scFv 8.3 and scFv 2.3.5, respectively, that had been mixed with an equal volume of mAb 9E10 (20 μg/ml) for 1 h at RT or for 24 h at 4 °C. Controls include purified scFv fragments that had not been mixed with mAb 9E10, anti-HLA-A2, A24, A28 mAb CR11-351, anti-c-myc mAb 9E10 and irrelevant scFv mixed with mAb 9E10 for 1 h (scFv 2.3.5 to HLA-A*0201-MART1$_{27-35}$, or scFv 8.3 to HLA-A*0201-HER-2/neu$_{369-377}$). Bound scFv fragment–mAb 9E10 complexes were detected with HRP-conjugated goat anti-mouse IgG (Fc specific) antibodies. Bound scFv fragments which had not been mixed with mAb 9E10 were detected by sequential incubation with mAb 9E10 and HRP-conjugated goat anti-mouse IgG (Fc specific) antibodies. TMB was used as a substrate. Results, expressed as absorbance at 450 nm, are the mean±standard deviation of the values generated from three independent experiments.

(Fig. 2C). The increase in reactivity of scFv 8.3 upon mixing with mAb 9E10 or b-9E10 did not result in an increase in nonspecific reactivity with either purified HLA-A*0201-HER-2/neu$_{369-377}$ complexes or HER-2/neu$_{369-377}$ pulsed T2 cells, respectively. No significant difference was observed in the



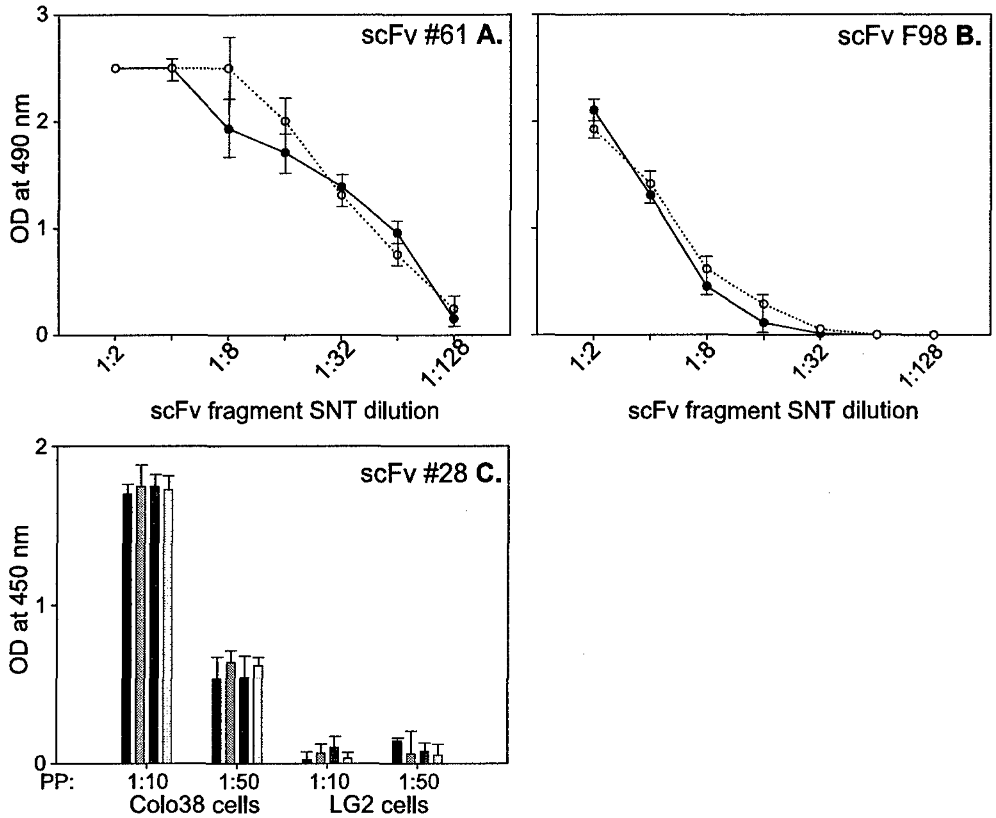


Fig. 3. Lack of detectable effect of mixing time with anti-tag mAb 9E10 on the reactivity of scFv fragments with target antigens. Cultured human melanoma cells Colo 38 and FO-1 (2×10^5/well) prepared on 96-well microtiter ELISA plates were incubated with 50 μl of serially diluted (A) scFv #61 SNT or (B) scFv F98 SNT which had been mixed with an equal volume of mAb b-9E10 in 2% BSA-PBS (0.5 μg/ml) in a well for 1 h (○) or 0 h (●) at RT. (C) Cells were incubated with 50 μl of 1:10 or 1:50 diluted scFv #28 PP pre-incubated with an equal volume of mAb b-9E10 in 2% BSA-PBS (0.5 μg/ml) in a well for 0 h (■), 1 h (▨), 4 h (▨), or overnight (■). Bound scFv fragment–mAb b-9E10 complexes were detected with HRP-conjugated streptavidin. Results, expressed as absorbance at 490 and 450 nm when *ortho*-phenylenediamine–H_2O_2 and TMB were used as substrates, respectively, are the mean±standard deviation of the values generated from three independent experiments is indicated.

reactivity of scFv 8.3 with the HLA-A*0201-$MART1_{27-35}$ complex whether it was mixed with mAb 9E10 for 1 or 24 h (Fig. 1), suggesting that scFv–mAb 9E10 complexes form rapidly. In contrast, scFv 2.3.5, which demonstrates high specific reactivity with the HLA-A*0201-HER-2/$neu_{369-377}$ complex and HER-2/$neu_{369-377}$ peptide pulsed T2 cells without mixing with mAb 9E10 and b-9E10,

Fig. 2. Increased reactivity of purified scFv fragments in flow cytometry following mixing with anti-tag mAb 9E10. $MART1_{27-35}$ and HER-2/$neu_{369-377}$ peptide pulsed T2 cells were incubated with (A) 1 μg scFv 8.3 or (B) 1 μg scFv 2.3.5, respectively, that had previously been mixed with an equal volume of mAb b-9E10 (20 μg/ml) for 1 h at RT (- - -). (C) The mean values±standard deviation of the results generated from three independent experiments with scFv fragments which had been mixed (■) or not mixed (▨) with mAb b-9E10 are indicated. Controls include purified scFv fragments that had not been mixed with mAb b-9E10 (middle panel), biotinylated anti-HLA-A2, A24, A28 mAb CR11-351 (—, open histogram) and anti-c-myc mAb b-9E10 (—, filled histogram). Bound scFv fragment–mAb b-9E10 complexes and biotinylated mAb were detected with PE-conjugated streptavidin. Bound scFv fragments which had not been mixed with mAb b-9E10 were detected by sequential incubation with mAb b-9E10 and PE-conjugated streptavidin. The binding was analyzed on FACScan (Becton-Dickinson) with CellQuest™ software (Becton-Dickinson). Results, expressed as mean fluorescence intensity (MFI), are the mean±standard deviation of the values generated from three independent experiments.

respectively (Figs. 1 and 2), did not demonstrate an increase in reactivity with its target antigen upon mixing with mAb 9E10 or b-9E10.

3.2. Lack of detectable effect of mixing time with anti-tag mAb 9E10 on the reactivity of scFv fragments with target antigens

Additional experiments tested the effect of length of mixing time of mAb 9E10 with scFv fragments on the increase in their reactivity with targets in binding assays. To this end, mAb b-9E10 (0.5 μg/ml) was mixed with scFv fragment PP and SNT. Subsequently, the mixture was immediately used in binding assays or incubated for 1, 4 and 24 h at RT prior to use in binding assays. Fig. 3 shows the mean value±standard deviation of the results generated from three independent experiments. As indicated, the maximal effect was already observed when scFv fragment preparations were mixed with mAb 9E10 and directly used in binding assays. No change was detected by prolonging mixing time to 24 h. This conclusion was applicable to scFv fragments isolated from the synthetic scFv library (#1) such as scFv #61 and scFv #28 (Fig. 3A and C) as well as those isolated from the large semi-synthetic phage display scFv library such as F98 (Fig. 3B). Therefore scFv fragment–mAb 9E10 mixtures were directly used in all subsequent experiments. Collectively, these findings suggest that the formation of scFv fragment–mAb 9E10 complexes is rapid and stable over time.

3.3. Increased titer of purified scFv fragment preparations in binding assays following mixing with anti-tag mAb 9E10

To determine if mixing anti-tag mAb 9E10 with scFv fragment preparations could increase their titer in ELISA, a purified scFv 8.3 preparation was titrated following mixing with mAb 9E10. Fig. 4 shows the mean value±standard deviation of the results generated from three independent experiments. As indicated in Fig. 4A, scFv 8.3, which reacts weakly with HLA-A*0201-MART1$_{27-35}$ complexes at high concentrations, showed on average a twofold increase in reactivity upon mixing with mAb 9E10. It is noteworthy that the increase in purified scFv fragment reactivity could be seen over all concentrations of purified scFv fragment tested. In contrast, scFv 2.3.5, which reacts strongly with HLA-A*0201-HER-2/neu$_{369-377}$ complexes, did not show increased binding to these

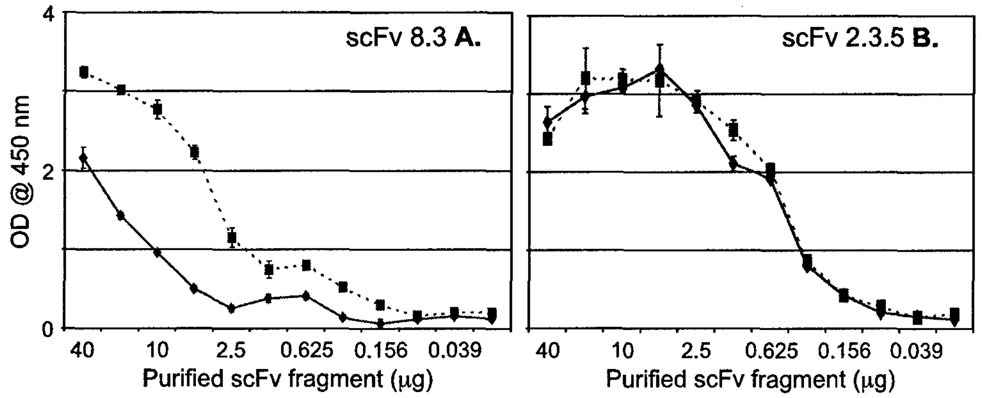


Fig. 4. Increased titer of purified scFv fragment preparations in ELISA following mixing with anti-tag mAb 9E10. Biotinylated HLA-A*0201-MART1$_{27-35}$ and HLA-A*0201-HER-2/neu$_{369-377}$ complexes (20 ng/well) were immobilized on a 96-well microtiter plate. Bound HLA-A*0201-MART1$_{27-35}$ and HLA-A*0201-HER-2/neu$_{369-377}$ complexes were incubated with (A) 1 μg scFv 8.3 and (B) 1 μg scFv 2.3.5, respectively, which had been mixed with an equal volume of mAb 9E10 (20 μg/ml) (--■--). Purified scFv fragments which had not been mixed with mAb 9E10 served as controls (—◆—). Bound scFv fragment–mAb 9E10 complexes were detected with HRP-conjugated goat anti-mouse IgG (Fc specific) antibodies. Bound scFv fragments which had not been mixed with mAb 9E10 were detected by sequential incubation with mAb 9E10 and HRP-conjugated goat anti-mouse IgG (Fc specific) antibodies. TMB was used as a substrate. Results, expressed as absorbance at 450 nm, are the mean±standard deviation of the values generated from three independent experiments.

complexes following mixing with 20 μg/ml of mAb 9E10 (Fig. 4B).

3.4. Increase in the number of antigen-reactive scFv fragments identified in a panel of clones isolated from phage display scFv libraries following mixing of scFv SNT with anti-tag mAb 9E10

Panning the synthetic scFv Library (#1) and the large semi-synthetic library with whole cells, proteins and carbohydrate antigens resulted in the isolation of seven panels of scFv fragments (Table 1). To determine if the formation of scFv fragment–mAb b-9E10 complexes could increase the yield of antigen-reactive scFv clones isolated by panning phage display antibody libraries with targets of interest, 40 clones from each of the seven panels of isolated scFv fragments were tested in ELISA with the corresponding antigen following mixing scFv fragment SNT with anti-c-myc mAb b-9E10. Simultaneously, the same 40 clones from each of the seven panels of isolated scFv fragments were tested in ELISA with the corresponding antigen without mixing of scFv fragment SNT with mAb b-9E10. As shown in Table 1, mixing scFv fragment SNT with anti-c-myc mAb b-9E10 resulted, on average, in a fivefold increase in the number of positive clones. It is noteworthy that in some cases antigen-reactive scFv fragments could not be detected without mixing scFv fragment SNT with anti-c-myc mAb b-9E10. The increase in the number of positive clones was not dependent on the library (synthetic scFv Library (#1) and the large semi-synthetic library) or antigen (whole cell, protein and carbohydrate antigen) used for panning. Representative results from four panels of isolated scFv fragments tested in ELISA with the corresponding antigen following mixing scFv fragment SNT with anti-c-myc mAb b-9E10 are shown in Fig. 5. All clones which demonstrated reactivity with their corresponding antigen in ELISA are indicated.

Table 1
Increase in the number of antigen-reactive scFv fragments identified in a panel of clones isolated from phage display Ab libraries following mixing with anti-tag mAb 9E10

Antigen	Phage library	Mixed	Non-mixed
Whole cells			
SK-MEL-28 cells	#1[a]	13/40[b] (33%)[c]	2/40 (5%)
Colo 38 cells	sspdl[d]	25/40 (63%)	10/40 (25%)
FO-1 cells	sspdl	35/40 (88%)	15/40 (38%)
Glycoprotein			
mAb TP61.5+Colo 38 lysate	#1	2/40 (5%)	0/40 (0%)
mAb CR11-351	#1	25/40 (63%)	0/40 (0%)
p97	#1	9/40 (23%)	1/40 (3%)
Carbohydrate			
$\alpha_{1,6}$ Dextran N279	#1	5/40 (13%)	1/40 (3%)
Total % positive clones:		127/280 (45%)	29/280 (10%)

Ninety-six-well microtiter ELISA plates were prepared with purified antigen or cells. ScFv fragment SNT (50 μl) from 40 clones isolated by panning a phage display scFv library were mixed with an equal volume of mAb b-9E10 in 2% BSA-PBS (0.5 μg/ml). The mixture was incubated with their corresponding antigens. Bound scFv fragment–mAb b-9E10 complexes were detected with HRP-conjugated streptavidin. *Ortho*-phenylenediamine–H_2O_2 was used as a substrate. Results are expressed as absorbance at 490 nm. Clones were considered positive if OD at 490 nm was greater than 0.5.

[a] Synthetic scFv library (#1).
[b] No. of positive clones/total no. of clones screened.
[c] Percentage of positive clones.
[d] Semi-synthetic phage display scFv library.

3.5. Increased titer of scFv fragment SNT and PP in binding assays following mixing with anti-tag mAb 9E10

To determine whether mixing unpurified scFv fragment preparations with mAb 9E10 could increase their titer with the corresponding antigen, several of the scFv fragment clones isolated from phage display antibody libraries were titrated with the corresponding antigen following mixing of scFv fragment SNT and PP with mAb b-9E10. The results were compared with the titers of the same scFv fragment SNT and PP tested without mixing with mAb b-9E10. Fig. 6 shows the mean value±standard deviation of the results generated from three independent experiments. As indicated, with all the scFv fragments tested, mixing scFv fragment SNT and PP with mAb 9E10 increased the titer of both scFv fragment SNT and PP. This increase ranged from 2- to 32-fold for the SNT tested and 4- to 8-fold for the PP tested. It is noteworthy that the enhancement of the titer was not associated with an increase in the nonspecific

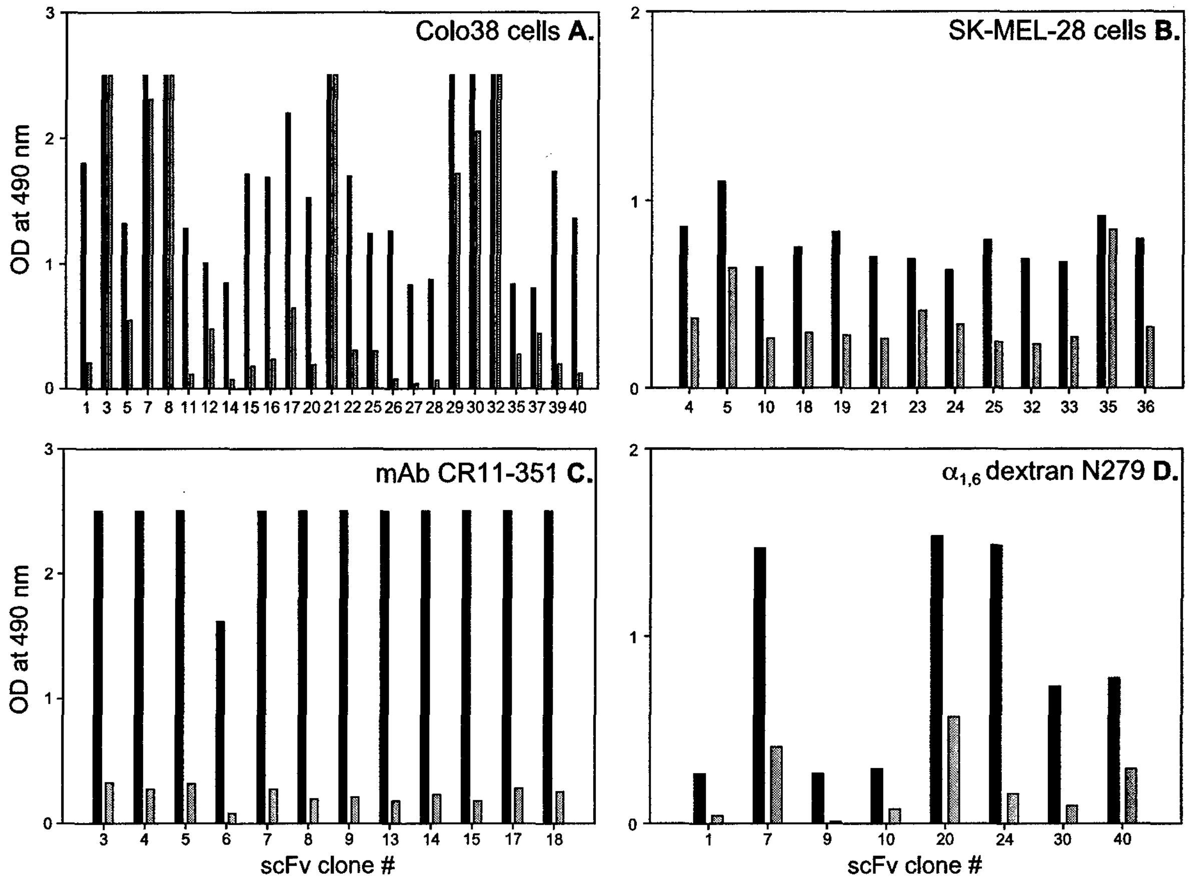


Fig. 5. Increased number of antigen-reactive scFv fragments identified in a panel of clones isolated from phage display scFv libraries following mixing with anti-tag mAb 9E10. Ninety-six-well microtiter ELISA plates were prepared with purified antigen or cells. Antigens and cells were incubated with 50 μl of scFv fragment SNT that had mixed with an equal volume of mAb b-9E10 2% BSA-PBS (0.5 μg/ml) were incubated with their corresponding antigens (■). ScFv fragment SNT which had not been mixed with mAb 9E10 served as controls (▒). scFv clones were isolated by panning the large semi-synthetic phage display library with Colo38 cells (panel A) or the synthetic phage display library (#1) with SK-MEL-28 cells (panel B), mAb CR11-351 (panel C) or $\alpha_{1,6}$ dextran N279 (panel D). Controls include the human lymphoid cell line LG2, isotype-matched mouse IgG1 mAb MK2-23 and BSA in the screening assays with Colo 38 cells, SK-MEL-28 cells, mAb CR11-351 and $\alpha_{1,6}$ dextran N279, respectively (data not shown). Bound scFv fragment–mAb b-9E10 complexes were detected with HRP-conjugated streptavidin. *Ortho*-phenylenediamine–H_2O_2 was used as a substrate. Results are expressed as absorbance at 490 nm.

binding of SNT and PP to cells which do not express the target antigen.

4. Discussion

The present study has shown that mixing scFv fragments with anti-tag mAb 9E10 markedly increases the sensitivity of binding assays to detect scFv fragments. Use of this method in binding assays resulted in a reproducible 2- to 32-fold increase in the reactivity of scFv fragment preparations with the corresponding antigens. Despite its increased sensitivity, use of this assay did not result in an increase in nonspecific scFv fragment reactivity. Moreover, the increase in the reactivity of scFv fragments with the corresponding antigen was observed over a broad range of scFv fragment (6–800 μg/ml) and mAb 9E10 (0.5–30 μg/ml) concentrations thereby facilitating the testing of scFv fragment preparations with unknown scFv fragment concentrations. In this regard, when screening clones isolated from phage display Ab

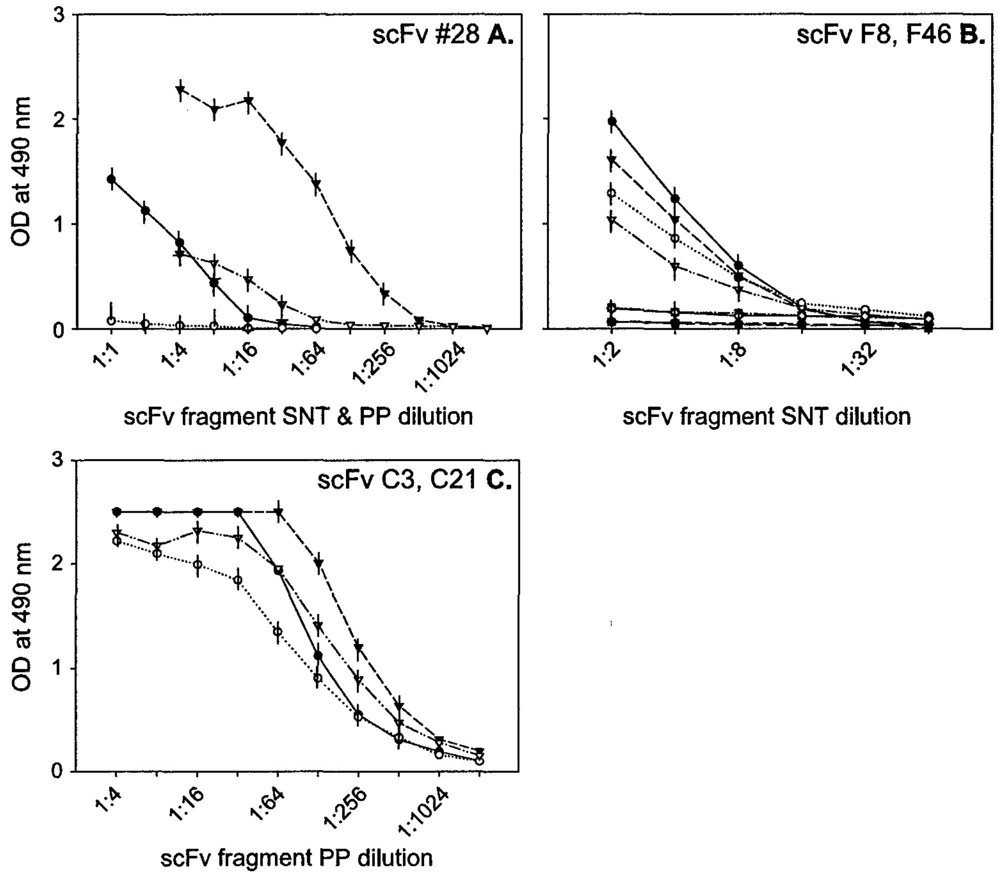


Fig. 6. Increased titer of scFv fragment SNT and PP preparations in ELISA following mixing with anti-tag mAb 9E10. Cultured human melanoma cells Colo 38 and FO-1 were added to a 96-well microtiter ELISA plate (2x10^5 cells/well) and then incubated with 50 μl of serially diluted scFv fragment SNT or PP which had been mixed with an equal volume of mAb b-9E10 2% BSA-PBS (0.5 μg/ml). (A) Titer of SNT (●) and PP (▲) of scFv #28. Controls include SNT (O) and PP (△) of scFv #28 which had not been mixed with mAb b-9E10 and cultured human lymphoid cells LG2 (data not shown). (B) Titer of SNT of scFv F8 (●) and scFv F46 (▲). Controls include SNT of scFv F8 (O) and scFv F46 (△) which had not been mixed with mAb b-9E10 and cultured human lymphoid cells LG2 (◇,□,◆,■). (C) Titer of PP of scFv C3 (●) and scFv C21 (▲). Controls include PP of scFv C3 (O) and scFv C21 (△) which had not been mixed with mAb b-9E10 and cultured human lymphoid cells LG2 (data not shown). Bound scFv fragment–mAb b-9E10 complexes were detected with HRP-conjugated streptavidin. *Ortho*-phenylenediamine–H_2O_2 was used as a substrate. Bound scFv fragments which had not been mixed with mAb b-9E10 were detected by sequential incubation with mAb b-9E10 and HRP-conjugated streptavidin. Results, expressed as absorbance at 490 nm, are the mean±standard deviation of the values generated from three independent experiments.

libraries mixing scFv fragment SNT with anti-tag mAb 9E10 for 0 h resulted in a reproducible fivefold increase in the number of antigen-reactive scFv fragments identified. Moreover, in some cases, antigen-reactive scFv fragments could not be detected without mixing scFv fragment SNT with anti-c-myc mAb b-9E10. Lastly, this method which could be applied in both ELISA and flow cytometry, is independent of the characteristics of the antigen (i.e. whole cells, carbohydrates and purified protein) and of the library (i.e. synthetic scFv Library (#1), a large semi-synthetic phage display scFv library and the human synthetic VH+VL scFv library (Griffin.1 library)) used.

Exploitation of the full range of scFv fragment specificities present in a phage display scFv library requires sensitive assays capable of detecting scFv fragments with a wide range of reactivities to the corresponding antigen. Mixing scFv fragments with anti-tag mAb 9E10 allows one to identify scFv fragments which display low reactivity with the target antigen, either because of their low affinity/avidity and/or low antigen density in the case of cellular antigens. The increased yield of antigen-reactive scFv clones and the titer of scFv fragment detection is likely to be attributable to the ability of anti-c-myc mAb 9E10 to dimerize scFv fragments. Thus, while the binding affinity of individual scFv fragments for their respective antigens cannot be altered by this mechanism, their avidity is increased due to the linkage of scFv fragments recognizing the same antigenic epitope. It is noteworthy that when assaying against immobilized antigens as in ELISA, the ability of scFv fragment multimers to bind simultaneously to separate target antigens is dependent on the proper spatial orientation of the multivalent binding sites. scFv fragment dimers, trimers, and tetramers have been shown to have linker flexibility (Hudson and Kortt, 1999). It is hypothesized that the dimerization of scFv fragments by anti-tag mAb also results in a structure which is not spatially rigid, such that the individual scFv fragments linked in this manner can independently bind to the immobilized target antigen or cell as well as the monomeric scFv fragments. As a result, mixing scFv fragments with anti-tag mAb can increase the sensitivity of binding assays to detect the specific reactivity of antigen-reactive scFv fragments.

An alternative method of increasing the reactivity of scFv fragments isolated from phage display scFv libraries is through scFv fragment oligomerization (e.g., dimerization, trimerization, or tetramerization) (Hudson and Kortt, 1999, Power and Hudson, 2000). The mechanism underlying the ability of scFv fragment oligomerization to increase the reactivity of scFv fragments is similar to that of mixing scFv fragment preparations with anti-tag mAb, in that it also relies on the increased avidity of the scFv antibody complex as a result of its multivalent nature. However, a limitation of scFv fragment oligomerization is that this approach can only be applied once scFv fragment have been isolated and characterized. The methodology described herein overcomes the need to first isolate and characterize scFv fragments prior to increasing their avidity, thereby facilitating the isolation of scFv clones with a broad range of affinities to antigens of interest from phage display scFv libraries.

In conclusion, the current study provides an efficient, sensitive, simple, and reproducible technique to enhance the specific reactivity of scFv fragments in binding assays. This method is suitable for use with a number of different libraries as well as antigens. It is expected that this method to detect scFv fragments will facilitate the use of phage display scFv libraries.

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Immune selection of hot-spot *B2M* gene mutations, HLA-A2 allospecificity loss and antigen processing machinery component downregulation in melanoma cells derived from recurrent metastases following immunotherapy[1]

Running title: Multiple HLA class I antigen defects in melanoma cells

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Abstract

Scanty information is available about the mechanisms underlying HLA class I antigen abnormalities in malignant cells exposed to strong T cell-mediated selective pressure. Here we have characterized the molecular defects underlying HLA class I antigen loss in five melanoma cell lines derived from recurrent metastases following initial clinical responses to T cell-based immunotherapy. Point mutations in the translation initiation codon (ATG→ATA) and in codon 31 (TCA→TGA) of the β_2-microglobulin (β_2m) gene were identified in the melanoma cell lines 1074MEL and 1174MEL, respectively. A hot-spot CT dinucleotide deletion within codon 13 to 15 was found in the melanoma cell lines 1106MEL, 1180MEL and 1259MEL. Reconstitution of β_2m expression restored HLA class I antigen expression in the five melanoma cell lines; however, the *HLA-A* and *HLA-B, -C* gene products were differentially expressed by 1074MEL, 1106MEL and 1259MEL cells. In addition, in 1259MEL cells the antigen processing machinery components calnexin, calreticulin, and LMP10 are downregulated and HLA-A2 antigens are selectively lost because of a single cytosine deletion in the *HLA-A2* gene exon 4. Our results in conjunction with those in the literature suggest the emergence of a preferential *B2M* gene mutation in melanoma cells following strong T cell-mediated immune selection. Furthermore, the presence of multiple HLA class I antigen defects within a tumor cell population may reflect the accumulation of multiple escape mechanisms developed by melanoma cells to avoid distinct sequential T cell-mediated selective events.

Introduction

In animal model systems, host's immune system has been convincingly shown to be able to mount an immune response against a naturally growing tumor (1). Recent evidence suggests that this immune response is not always beneficial since it may reshape a tumor cell population by favouring the outgrowth of tumor cells which have developed immunoresistance mechanisms. This phenomenon is referred to as immunoediting (2). In this regard, the current revival of the cancer immune surveillance theory has emphasized the role of immune selective pressure in the generation of tumor immune escape variants in the course of a malignant disease (2). Among the several immune effector mechanisms that may impose selective pressure on tumor cells, cytotoxic T lymphocytes (CTL)[3] are believed to be the major players because of their postulated crucial role in the control of tumor growth. Since the recognition of tumor cells by CTL is mediated by MHC class I antigens presenting tumor antigen (TA)-derived peptides (1,3), the immune selective pressure imposed by CTL on tumor cell populations may lead to the isolation and expansion of tumor cell subpopulations with impaired MHC class I antigen-TA–derived peptide complex expression. In humans, this possibility is supported, in part, by the frequent identification of malignant lesions with HLA class I antigen abnormalities (4) that are likely to have escaped recognition and destruction by HLA class I antigen-restricted, TA-specific CTL as a result of continuous CTL-mediated immune selective pressure. The negative impact of this phenomenon on the clinical course of the disease is implied by the correlation between HLA class I antigen abnormalities in tumor lesions and a poor clinical outcome in various types of malignant diseases (4).

Total HLA class I antigen loss by tumor cells results in their complete resistance to recognition and destruction by CTL (5). The mechanism underlying this abnormal phenotype has been frequently found to be loss of β_2-microglobulin (β_2m), which is required for the formation of the HLA class I heavy chain-β_2m-peptide complex and its transport to the cell surface (6,7). Two genetic events, namely, mutations of one copy of the *B2M* gene and loss of the other copy, i.e. loss of heterozygosity (LOH), have been found to underlie β_2m loss in malignant cells, although the chronological sequence of the two events is not known (7). Several types of *B2M* gene mutations ranging from large deletions, which abolish transcription, to point mutations, which in most cases abolish translation, have been described (7). However, it is not clear whether a preferential *B2M* gene mutation would arise as a result of strong and continuous T cell selective pressure. To test this hypothesis, we set out to characterize the genetic defects underlying the β_2m loss previously identified in the five melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL (8). These melanoma cell lines were chosen since they were derived from recurrent metastases in patients who had initially experienced clinical responses to T cell-based immunotherapy (8). Therefore, they are likely to have been exposed to strong T cell selective pressure. In addition, we tested whether a host's immune system counteracts escape mechanisms developed by tumor cells by changing the target of its immune response. Lastly, we tested whether the change in the specificity of the selective pressure imposed on a tumor cell population results in the outgrowth of a tumor cell population which has accumulated multiple escape mechanisms to counteract changes in the specificity of the host's immune response. To this end, we determined whether β_2m loss is associated with additional abnormalities in molecules involved in HLA class I-associated antigen processing and peptide presentation in the five melanoma cell lines.

Materials and Methods

Cell lines. Cultured human melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL were derived from recurrent metastases in five patients who had experienced clinical responses to T cell-based immunotherapy (8). These cell lines, the melanoma cell lines Colo38 and FO-1 and the lymphoblastoid cell line LG-2 were maintained in RPMI 1640 medium (Tissue Culture Media Facility, Roswell Park Cancer Institute, Buffalo, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (ICN, Costa Mesa, CA) or with serum plus (Hazelton Biologics, Inc., Lenexa, KS) in a 5% CO_2 atmosphere at 37 °C. Patients 1074, 1106, 1174, 1180, and 1259's HLA class I phenotypes, as determined by typing of peripheral blood mononuclear cells, are A2/A3, B7/B62, Cw3/-; A11/A31, B38/-, -/-; A26/A33, B14/B37, Cw6/w7; A25/A25, B14/B18, -/-; and A1/A2, B8/B44, Cw5/w6, respectively.

Monoclonal and polyclonal antibodies. The mouse monoclonal antibodies (mAb) W6/32, which recognizes β_2m-associated HLA-A, -B, -C, -E, and -G heavy chains (9,10); mAb LGIII-147.4.1, which recognizes β_2m-associated HLA-A heavy chains, excluding –A23, -A24, -A25, -A32 (11); mAb B1.23.1, which recognizes β_2m-free and -associated HLA-B and -C heavy chains (12); anti-HLA-A2, -A24, -A28 mAb CR11-351 (13,14); anti-HLA-A2, -A28 mAb KS-1 (14); anti-HLA-B7, -B27, -Bw42, -Bw54, -Bw55, -Bw56, -Bw67, -Bw73 mAb KS-4 (15); mAb HCA-2, which recognizes β_2m-free HLA-A (excluding -A24), -B7301, and -G heavy chains (16,17), mAb HC-10, which recognizes β_2m-free HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, -A33, -B (excluding -B5702, -B5804, and -B73) heavy chain (16-18); anti-β_2m mAb L368 (19); anti-tapasin mAb TO-3 (20); anti-calnexin mAb TO-5 (20); anti-ERp57 mAb TO-2 (20); anti-calreticulin mAb TO-11 (20); anti-TAP1 mAb NOB-1 (S. Ferrone, unpublished results); anti-

20S proteasome constitutive β subunits delta, MB1 and Z mAb SY-5, SJJ-5, and NB-1, respectively (20 and unpublished results); anti-20S proteasome inducible subunits low molecular weight polypeptide (LMP)2, LMP7, and LMP10 mAb SY-1, HB-2, and TO-7, respectively (20); and anti-HLA-DP, -DQ, -DR mAb Q5/13 (21) were developed and characterized as described. The anti-idiotypic mAb MK2-23, IgG_1 (22) and F3C25, IgG_{2a} (23) were used as isotype-matched irrelevant controls. R-phycoerythin (R-PE)-conjugated $F(ab')_2$ fragments of goat anti-mouse Fc and horseradish peroxidase (HRP)-conjugated goat anti-mouse Fc antibodies were purchased from DAKO (Carpinteria, CA) and Jackson Immunoresearch Laboratories (West Grove, PA), respectively.

HLA-A*0201-melanoma antigen recognized by T cells $(MART)1_{27\text{-}35}$ complex-specific scFv tetramers. The monomeric HLA-A*0201-$MART1_{27\text{-}35}$–specific scFv 8.3 was isolated from the human synthetic V_H+V_L scFv library (Griffin.1 library) by panning with HLA-A*0201-$MART1_{27\text{-}35}$ peptide complexes (Campoli et al., manuscript in preparation). To construct tetrameric scFv 8.3, monomeric scFv 8.3 were engineered with a carboxyl-terminal BirA biotinylation tag (scFv 8.3-BirA) and expressed from ampicillin-resistant bacterial colonies as previously described (24-26). scFv 8.3-BirA was then purified with Ni-affinity chromatography (27) following the manufacturer's instructions. The purity of scFv 8.3-BirA was monitored by SDS-PAGE and silver staining. Tetrameric scFv 8.3 complexes were generated by linking monomeric scFv 8.3-BirA with streptavidin-PE as described (28).

Interferon (IFN)-γ and synthetic oligonucleotide primers. Recombinant human IFN-γ was purchased from Roche, Inc. (Nutley, NJ). Oligonucleotide primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). They include *B2M* gene-specific primers *261M*, 5'-CCTGAAGCTGACAGCATTCG-3', *262M*, 5'-ACCTCCATGATGCTGCTT ACA-3',

744M, 5'-CTCTAACCTGGCACTG CGTCGC-3', *491M*, 5'-CTGGCAATATTAATGTGTCTTTCC-3', *468M*, 5'-TTGAGAAGGAAGTCACGGAGCG-3', and *462M*, 5'-TCATACACAACTTTCAGCAGC-3'; *HLA-A* or *HLA-A*0201*gene-specific primers 5pE1A2, 5'-TCCTGCTACTCTCGGGGGCT-3', 5pE2A, 5'-GACGCCGCGAGCCAGAGGAT-3', AP2, 5'-TCACTTTCCGTGCTCCCC-3', 3pE3A2, 5'-CTCCCACTTGTGCTTGGTGG-3', and 3pE8A, 5'-AGTCACAAAGGGAAGGGCAGG-3', A2E3-5', 5'-GGAGCAGTGGAGAGC-3', A2E4-3', 5'-GGTGTATCTCTGCTCC-3'; and β–actin (*ACTB*) gene-specific primers sense 5'-GACTTCGAGCAAGAGATGGCCAC-3' and antisense 5'-CAATGCCAGGGTACATGGTGGTG-3'.

Flow cytometry. Flow cytometric analysis of cells for HLA class I antigen expression was performed as described (5). Briefly, cells (1×10^5) were incubated for 1 hr at 4°C with primary mouse mAb at concentrations of 5-10 μg/ml before incubation for 30 min at 4°C with an optimal amount of R-PE-conjugated, mouse Fc-specific goat IgG $F(ab')_2$ fragments (DAKO). After washing off excess antibodies, cells were fixed with 0.5% paraformaldehyde (Sigma, St. Louis, MO) in PBS and analyzed on FACScan (Becton-Dickinson, San Jose, CA) with CellQuest™ software (Becton-Dickinson). Results are expressed as fold increase of mean fluorescence intensity over the isotype-control background (Fold MFI). Intracellular antigen processing machinery (APM) component, β_2m and HLA class I heavy chain expression was analyzed as previously described (29). Briefly, cells (1×10^5) were fixed with 2% paraformaldehyde (Sigma) in PBS, heat-denatured with microwave and permeabilized with 0.1% saponin in 0.5% BSA/PBS before incubation with primary mAb. Following three washes, cells were stained with an optimal amount of R-PE-conjugated $F(ab')_2$ fragments of goat anti-mouse Fc antibodies (DAKO) and analyzed on FACScan (Becton-Dickinson) with CellQuest™ software (Becton-Dickinson).

Results are expressed as Fold MFI. Melanoma cells were stained with the HLA-A*0201-$MART1_{27\text{-}35}$ complex-specific scFv 8.3 tetramer by incubating 1×10^6 cells (5×10^7 cells/ml) with the scFv 8.3 tetramer (10μg) for 1hr at 4°C. After three washes with 0.5% BSA/PBS, cells were resuspended in 100 μl of PBS, fixed in 300 μl of 2% formaldehyde/PBS and analyzed by flow cytometry. Results are expressed as Fold MFI.

Western blotting. Western blot analysis of cell lysates for APM component, β_2m and HLA class I heavy chain expression was performed as described (5). Briefly, cells (1×10^7) were solubilized in 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris, pH7.4, 150 mM NaCl, 5 mM EDTA) in the presence of a cocktail of protease inhibitors (Calbiochem, San Diego, CA) and protein concentration was measured by Bradford assay (Bio-Rad, Hercules, CA). Twenty μg of total protein were separated by SDS-PAGE, semidry-transferred (Bio-Rad) to PVDF membranes (Millipore, Billerica, MA) and blocked with 5% dry-milk/PBS before incubation with primary mAb (5~50 μg/ml) overnight at 4°C. After washing off excess primary mAb, membranes were incubated with an optimal amount of HRP-conjugated goat anti-mouse Fc antibodies for 30 min at room temperature. Antigens were revealed by the ECL system (Amershan, Piscataway, CA).

PCR and sequence analysis. Total RNA isolation, first-strand cDNA synthesis, and reverse-transcription (RT)-PCR were carried out as described elsewhere (5). Genomic DNA was extracted and gene fragments were amplified by PCR as described elsewhere (5). PCR products were resolved by 1.5% agarose electrophoresis and purified with a QIAquick gel extraction kit (Qiagen Inc., Valencia, CA.). Direct sequencing of purified PCR products was performed using an ABI-PRISM model 377 sequencer (PE Applied Biosystems, Foster City, CA). Alternatively, PCR products were cloned with a pCR II-TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced with T7 and Sp6 primers.

Transfection. Cells were transfected with a wild-type *B2M* gene or cDNA utilizing lipofectamine-mediated gene transfer (Invitrogen) according to the manufacturer's instructions. Briefly, pb2m13 (30), pcDNA3-b2m (5) or the empty plasmid pcDNA3-neo (Invitrogen) was mixed with Lipofectamine^{TM} 2000 before being added to melanoma cells grown in monolayers with a 90% confluence. Cells were selected 2 days after transfection in medium containing G418-sulfate (1mg/ml) (Calbiochem). After 2~3 weeks of selection, G418-resistant clones were picked, expanded and screened by flow cytometry for HLA class I antigen expression. Positive clones were then further expanded in complete media supplemented with a maintenance dose (0.3mg/ml) of G418.

Results

Identification of B2M *gene mutations in the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL.*

As shown in Fig. 1A, HLA class I antigens are not detectable on the surface of the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL, since the five cell lines are not stained by anti-HLA class I mAb W6/32. Furthermore, β_2m protein was not detected in the lysates of the five HLA class I$^-$ melanoma cell lines (Fig. 1B) although their *B2M* gene was transcribed (Fig. 1C). To test whether mutations in the coding region of the *B2M* gene exist, we purified and sequenced the RT-PCR-amplified *B2M* cDNA fragments obtained from the five HLA class I$^-$ melanoma cell lines. Analysis of the nucleotide sequences of the *B2M* cDNA identified mutations in each of the five HLA class I$^-$ melanoma cell lines. Specifically, as shown in Fig. 2, a G was replaced with an A (ATG→ATA) at the initiation codon of the *B2M* gene in 1074MEL cells. This mutation is expected to abolish the initiation of translation of the

B2M mRNA. In the cell line 1174MEL, a C was replaced with a G (TCA→TGA) at codon 31, resulting in the introduction of a premature TGA stop codon. In the cell lines 1106MEL, 1180MEL and 1259MEL, one CT dinucleotide (delCT) was deleted within the 8-bp CT repeat region of codons 13-15 in exon 1. This mutation causes a reading frame shift, resulting in 40 mis-sense codons followed by the introduction of an early stop at codon 55.

The mutations found in the *B2M* cDNA fragments in the five melanoma cell lines analyzed were confirmed at the genomic DNA level, since identical mutations were detected in the *B2M* genes in these five cell lines (Fig. 3). These mutations are the only ones present in their *B2M* genes, since sequencing of PCR products corresponding to the 5' flanking region, the entire coding region and the 3' non-translation region of the genes detected no additional mutations (data not shown). It is noteworthy that three of the five cell lines analyzed were found to have the same delCT mutation in the *B2M* gene, suggesting the presence of a mutational hot spot in that region of the gene.

Differential expression of HLA-A and HLA-B, -C gene products on the melanoma cell lines 1074MEL, 1106MEL, and 1259MEL transfected with a wild-type B2M *gene or cDNA*

Transient reconstitution of β_2m expression has been previously shown to restore HLA class I antigen expression by the five melanoma cell lines (8). Furthermore, in preliminary experiments, we have found that the gene products of *HLA-A, HLA-B, and -C* loci were differentially expressed on 1074MEL, 1106MEL and 1259MEL cells, but not on 1174 MEL and 1180MEL cells (unpublished observations). To corroborate these findings, in the present study, we have established stable β_2m transfectants for 1074MEL, 1106MEL and 1259MEL melanoma cells and analyzed their HLA class I antigen expression. As shown in Fig. 4, HLA class I antigens were highly expressed on clones of β_2m-transfected 1074MEL (1074MEL.β_2.7), 1106MEL (1106MEL.β_2), and 1259MEL (1259MEL.β_2.18) cells, but were not detected on mock-transfected 1074MEL, 1106MEL, and 1259MEL cells. Upon stimulation with IFN-γ (300 U/ml) for 48 hrs at 37 °C, the level of total HLA class I antigens was increased only on the β_2m transfectants.

As shown in Fig. 5A, the gene products of *HLA-A, -B*, and *–C* loci are differentially expressed by the stable β_2m transfectants of 1074MEL, 1106MEL and 1259MEL melanoma cells. Flow cytometric analysis of cells stained with HLA-A-specific mAb LGIII147.4.1 and HLA-B and –C-specific mAb B1.23.1 detected a higher level of HLA-A antigens than of HLA-B, -C antigens on 1074MEL.β_2.7 and 1259MEL.β_2.18 cells, and a lower level of HLA-A antigens than of HLA-B, -C antigens on 1106MEL.β_2 cells. Following incubation with IFN-γ (300 U/ml) for 48 hrs at 37 °C, the downregulated HLA-A antigens on 1106.MEL.β_2 cells increased 3.5 fold, reaching a level comparable to the basal HLA-B and -C antigen level, while the downregulated HLA-B and -C antigens on 1074MEL.β_2.7 and 1259MEL.β_2.18 cells increased 3.7 and 3.1 fold, respectively, remaining at a lower level than that of HLA-A antigens under basal conditions (Fig. 5A).

Differential HLA-A and HLA-B, -C heavy chain expression in the melanoma cell lines 1074MEL, 1106MEL, and 1259MEL

We next tested whether the differential HLA-A, and -B, -C allospecificity expression on the cell membrane of β_2m-transfected 1074MEL, 1106MEL, and 1259MEL cells is correlated with differential expression of the corresponding HLA class I heavy chains. As shown in Fig. 5B, using flow cytometry with intracellular staining, we found that HLA-B, -C heavy chains were expressed at a considerably lower level than HLA-A heavy chains in 1074MEL and 1259MEL cells, but at a higher level than HLA-A heavy chains in 1106MEL cells. On the other hand, HLA-A, -B, and -C heavy chains were equally well expressed in 1174MEL and 1180MEL cells. The results obtained with flow cytometry were corroborated by Western blot analysis with HLA class I heavy chain-specific mAb of the lysates of the five melanoma cell lines (Fig. 5C and data not shown).

Selective HLA-A2 antigen loss caused by a single-base deletion in exon 4 of the HLA-A2 *gene in 1259MEL cells*

To test whether the differential expression of the gene products of both *HLA-A* and *–B* loci was detectable at the level of individual allospecificities, we analyzed HLA-A2 antigen expression on 1074MEL.β_2.7 cells (patient's HLA-A phenotype: A2/A3) and 1259MEL.β_2.18 cells (patient's HLA-A type: A1/A2), as well as HLA-B7 antigen expression on 1074MEL.β_2.7 cells (patient's HLA-B phenotype: B7/B62), with HLA-A2, -A24, -A28-specific mAb CR11-351, HLA-A2, -A28-specific mAb KS-1, and HLA-B7 cross-reacting group-specific mAb KS-4. While HLA-B7 antigens were expressed, although at a low level, on 1074MEL.β_2.7 cells (data not shown), to our surprise, HLA-A2 antigens were not detectable on 1259MEL.β_2.18 cells (Fig. 6). HLA-A2 antigens were not detected even following incubation with IFN-γ (300 U/ml) for 48 hrs at 37 °C (Fig. 6). The lack of HLA-A2 antigen expression by IFN-γ-treated 1259MEL.β_2.18 cells is not due to their unresponsiveness to the cytokine, since HLA-A, -B, and-C antigens (Fig. 5A) as well as HLA-DP, -DQ, and -DR antigens (Fig. 6) were upregulated or induced upon IFN-γ stimulation. These results suggest that HLA-A2 antigens were selectively lost in 1259MEL cells. Additional experiments investigated *HLA-A2* mRNA expression in 1259MEL cells. RT-PCR analysis detected bands with sizes similar to those found in the wild-type *HLA-A2* mRNA isolated from 1074MEL cells (Fig. 7A & 7B). Nucleotide sequence analysis of 1259MEL *HLA-A2* cDNA and gene fragments identified a single cytosine deletion at nucleotide 701 (delC701) in the exon 4 (Fig. 7C and data not shown). This mutation causes a reading-frame shift, resulting in the introduction of 5 mis-sense codons (234 to 238), followed by a premature stop codon TGA (Fig. 7D). The disrupted translation of *HLA-A2* mRNA is expected to result in the loss of most of the HLA-A2 heavy chain $\alpha 3$ domain. The latter has been shown by the crystal structure of a HLA-A2-β_2m-peptide complex (Fig. 8, reconstructed from Protein Data Bank ID 1AKJ) (31) to represent the major contact region for β_2m.

Heterogeneous antigen processing machinery (APM) component expression in the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL

APM components play a critical role in the generation of peptides to be loaded onto HLA class I antigens in cells (32) and have been found to be frequently downregulated in a variety of tumor types (33). Therefore, we analyzed the five melanoma cell lines for possible defects in the APM components, which include the endoplasmic reticulum chaperones calnexin, calreticulin, ERp57, and tapasin, the peptide transporter TAP1, the constitutive proteasome subunits delta, MB1, and Z, and the IFN-γ inducible proteasome subunits LMP2, LMP7, and LMP10. As shown in Fig. 9A, flow cytometric analysis of melanoma cells intracellularly stained with APM component-specific mAb detected all of these components at comparable levels in the five melanoma cell lines tested, except for a marked calnexin, calreticulin and LMP10 downregulation in 1259MEL cells. Of the latter three molecules, only LMP10 expression can be slightly restored by the administration of IFN-γ. The results obtained with flow cytometry were corroborated by Western blot analysis of the five cell line lysates with APM component-specific mAb (Fig. 9B).

*HLA-A*0201-MART1$_{27\text{-}35}$ complex expression on β_2m-transfected 1074MEL cells*

To determine the functionality of APM in melanoma cells analyzed in this study, we assessed HLA-A*0201-MART1$_{27\text{-}35}$ peptide complex expression by 1074MEL cells. As shown in Fig. 10, β_2m-transfected 1074MEL cells were stained by the HLA-A*0201-MART1$_{27\text{-}35}$ peptide complex-specific scFv8.3 tetramer. The staining is specific since HLA-A*0201-MART1$_{27\text{-}35}$ peptide complexes were detected on β_2m-transfected 1074MEL cells, but not on mock-transfected 1074MEL cells, β_2m-transfected 1106MEL cells (HLA-A11/A31), mock- and β_2m-transfected 1259MEL cells (HLA-A1/A2^{delC701}) and LG2 cells (HLA-A2^{+}). The expression of HLA-A*0201-MART1$_{27\text{-}35}$ peptide complexes on β_2m-transfected 1074MEL cells is compatible with their fully functional APM capable of generating HLA class I antigen-restricted, melanoma antigen (MA)-derived peptides.

Discussion

Taking advantage of a panel of β_2m-, HLA class I heavy chain- and APM component-specific mAb, we have performed a comprehensive analysis of all the molecules known to be involved in functional HLA class I antigen expression in five melanoma cell lines exposed to strong T cell selective pressure. Multiple molecular defects which compromise the presentation of MA-derived peptides to HLA class I antigen restricted CTL do exist in at least three of the five melanoma cell lines tested. These findings thus provide for the first time in the human system the molecular mechanisms underlying the described immunoediting phenomenon (2).

Three types of *B2M* gene mutations were identified in the five melanoma cell lines, all of which do not affect the transcription but result in lack of translation of the β_2m protein. The mutation harbored by the cell line 1074MEL (ATG to ATA) is novel, since it is different from two other initiation codon mutations identified in the Burkitt lymphoma cell line Daudi (ATG to ATC) (34) and in the melanoma cell line LB1622-MEL (ATG to AAG) (35). The non-sense mutation identified in the cell line 1174MEL (TCA to TGA), which introduces a premature stop in codon 31 of exon 2 of the *B2M* gene ($31^{Ser\rightarrow Stop}$), is identical to the recently described mutation in the melanoma cell line BB74-MEL (35). It is noteworthy that both LB1622-MEL and BB74-MEL cell lines were derived from lesions in patients who had undergone T cell-based immunotherapy, suggesting that these cells have been exposed to strong T cell selective pressure. On the other hand, the $31^{Ser\rightarrow Stop}$ mutation did not underlie the HLA class I antigen loss by other five melanoma cell lines originated from patients who had not been treated with T cell-based immunotherapy. Interestingly, the delCT mutation identified in the cell lines 1106MEL, 1180MEL and 1259MEL is present in three of five melanoma cell lines originated from patients receiving T cell-based immunotherapy but has only been described in one (Me1386) (5) out of five melanoma cell lines established from patients not treated with T cell-based immunotherapy. Taken together, these findings clearly suggest an association between the preferential selection of a *B2M* gene mutational hot spot (especially delCT) and T cell-based immunotherapy in melanoma cells. If confirmed by the analysis of a large number of melanoma cell lines, this association may suggest a relationship between the outgrowth of melanoma cells harboring certain types of genomic instability and the type/extent of immune selective pressure introduced by T cell-based immunotherapy. Whether the same rule applies to other types of tumors remains to be determined. In this regard, the delCT mutation in the *B2M* gene has been found only in two colorectal carcinoma cell lines with HLA class I antigen loss (36,37). As far as we know, the two patients from whom the two cell lines had been originated had not been treated with T cell-based immunotherapy. Whether the two patients had developed a strong T cell immunity against their own tumors is not known.

In melanoma, like in many other tumor types, LOH was found to associate with *B2M* gene mutation (7). This phenomenon also applies to the melanoma cell lines we have analyzed in this study, since no wild-type *B2M* gene sequence was found to superimpose the mutated ones. The cause of LOH of *B2M* gene is not known but is probably an interstitial deletion because of a mitotic recombination event (38).

Reconstitution of β_2m expression following transfection with a wild-type *B2M* gene or cDNA was sufficient to restore total HLA class I antigen expression on the five melanoma cell lines. These results confirm the pivotal role of β_2m in HLA class I antigen expression and show that β_2m defects represent the only mechanism underlying HLA class I antigen loss by the five melanoma cell lines we have analyzed. However, differential expression of the gene products of *HLA-A* and *HLA-B, -C* loci was found in the β_2m-transfected cell lines 1074MEL, 1106MEL, and 1259MEL. This phenotype, which is correlated with the differential HLA-A and HLA-B, -C heavy chain downregulation, appears to be caused by abnormalities in regulatory mechanisms, since transcription of the corresponding HLA class I alleles is downregulated but can be restored by the administration of IFN-γ. This possibility is supported by the lack of locus-specific trans-acting factors which results in selective HLA class I locus downregulation, as has been reported in melanoma cell lines as well as in colorectal carcinoma cell lines (7,39).

The frame-shift mutation identified in the *HLA-A2* gene exon 4 in 1259MEL cells is novel since it is different from those described in the literature. The latter include a large deletion from the 5'-flanking region to exon 4 and loss of one copy of chromosome 6 in the melanoma cell lines 970604 and 950822, respectively (40), as well as a 5'-flanking region deletion of the gene, chromosome loss and exon skipping in the melanoma cell lines SK-MEL-29.1.22, SK-MEL-29.1.29 and 624MEL28, respectively (41,42). In the case of 1259MEL cells, it remains to be determined whether the truncated HLA-A2 heavy chain can still be synthesized and fold properly to associate with β_2m and peptides and even secreted by 1259MEL cells. Our findings highlight the importance to analyze tumor cells for polymorphic HLA class I allospecificity expression and not to rely on the use of mAb recognizing monomorphic and locus-specific determinants. In the latter cases, abnormalities in a given HLA class I locus or an individual allele will not be found, because they are masked by the positive staining of the gene products encoded by any of the remaining loci.

Only in 1259MEL cells the APM components calnexin, calreticulin and LMP10 were found to be markedly downregulated. The reduced APM component expression did not appear to have functional implications, since β_2m-restored 1259MEL cells were still sensitive to lysis by H2 K^b-restricted, vaccinia virus-specific murine CTL upon transient transduction of H-2 K^b molecules, as were the other four melanoma cell lines (8). These results are consistent with those reported in the literature that do not support a critical role for calnexin, calreticulin and LMP10 in the presentation of MHC class I antigen-restricted peptides. However, the data obtained with the viral system may not be applicable to the presentation of MA-derived peptides and their role in eliciting CTL responses since, (i) the amount of MA synthesized in cells is not likely to be comparable to that of viral antigens produced in cells; and (ii) the T cell repertoire for viral antigens is not likely to be the same as that for MA. It remains to be determined whether β_2m-transfected 1259MEL cells are sensitive to lysis by autologous, MA-specific CTL. On the other hand, the detection of HLA*0201-MART1$_{27-35}$ peptide complexes by the β_2m-transfected HLA-A2$^+$ 1074MEL cells (1074MEL.β2.7) suggests that 1074MEL cells carry a functional APM capable of generating HLA class I antigen-restricted, MA-derived peptides. Furthermore, this result parallels our previous finding that β_2m-transduced 1074MEL cells were susceptible to lysis by HLA-A2 antigen-restricted, MA-specific CTL (8).

B2M gene and HLA class I heavy chain abnormalities were both present in 1259MEL cells, raising the question of their chronological sequence of appearance. In light of immune system's ability to reshape tumor cell populations over time (2), we favor the possibility that the multiple HLA class I antigen defects found in 1259MEL cells may have developed sequentially in the order of HLA-A2 antigen loss, HLA-B and –C antigen downregulation and β_2m loss, although the first two can also occur in the reverse order. The *B2M* gene mutation is postulated to be a late event. In this situation, HLA class I heavy chain abnormalities would provide 1259MEL cells with a mechanism to escape lysis by CTL which recognize immunodominant epitopes before a complete HLA class I-loss phenotype is obtained. On the other hand, if total HLA class I antigen loss caused by β_2m loss occurs early, abnormalities in heavy chains would not be advantageous to β_2m-deficient 1259MEL cells because they are not expected to be recognized by CTL. If our interpretation is correct, β_2m loss is also likely to be a late event in 1074MEL and 1106MEL cell lines, since both cell lines have HLA class I heavy chain downregulation in addition to *B2M* gene mutations.

Disease progression in patients with melanoma associated with HLA class I antigen loss in metastases raises the question of why tumor growth is not controlled by NK cells *in vivo*, given the increased *in vitro* and *in vivo* sensitivity to NK cell-mediated lysis of target cells which have lost MHC class I antigen expression (43). One can postulate at least two possible underlying mechanisms: either NK cells have an impaired migratory/effector function *in vivo* or the melanoma cells lack expression of molecules that activate NK cells. The latter possibility is supported by the markedly reduced sensitivity *in vitro* to NK cell-mediated lysis of 1074MEL and 1106MEL cells which express low levels of NK cell-activating ligands MHC class I chain related (MICA) and UL16-binding proteins (44). These findings thus provide an explanation for the lack of control of tumor cells with HLA class I antigen loss by NK cells *in vivo*. In contrast, the melanoma cell lines 1174MEL and 1259MEL are highly sensitive to NK cell-mediated lysis, which is correlated with a high level of MICA expression on the cell surface (44). Therefore, NK cells may have failed to migrate to the lesions from which the cell lines 1174MEL and 1259MEL were derived.

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Footnotes

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[3]Abbreviations used in this paper:

APM, antigen processing machinery; ; β_2m, β_2-microglobulin; CTL, cytotoxic T lymphocyte; FCS, fetal calf serum; HLA, human leukocyte antigen; HRP, horsersdish peroxidase; IFN-γ, interferon-γ; LMP, low molecular mass polypeptide; LOH, loss of heterozygosity; MA, melanoma antigen; mAb, monoclonal antibody; MART, melanoma antigen recognized by T cells; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MICA, MHC class I chain related A; NK, natural killer; R-PE, R-phycoerythin; RT, reverse transcription; scFv, single chain antibody fragment; TA, tumor antigen; TAP, transporter associated with antigen processing.

Figure legends

Figure 1. β_2m loss by the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. (A) Lack of HLA class I antigen expression by the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. Cells were stained with the mAb W6/32 (opened histogram) and analyzed by flow cytometry. The melanoma cell line Colo38 and the isotype-matched mAb F3C25 (closed histogram) were used as controls. (B) Lack of β_2m protein expression in the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. Lysates prepared from control- and IFN-γ-treated (300U/ml, 48hrs at 37°C) melanoma cells were tested with the mAb L368 in Western blotting. A lympoblastoid cell LG-2 lysate was used as a positive control. (C) *B2M* mRNA expression by the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. Total RNA was isolated from cells, reversely transcribed to first-strand cDNA, and subjected to PCR analysis with *B2M*- and *ACTB*-specific primers. PCR products were run on an agarose gel and visualized by ethidium bromide staining. Colo38 and FO-1 (45) melanoma cells were used as a positive and negative control, respectively.

Figure 2. Nucleotide and deduced amino acid sequence of *B2M* cDNA isolated from the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. Sequence conservation and non-translated codons are indicated by dashes and dots, respectively. Point mutations and CT deletions are indicated by bold letters and small upward arrows (^), respectively. Exon-exon junctions are indicated by closed downward triangles (▼). Consensus *B2M* cDNA sequence was obtained from the NCBI GenBank (accession no. BC06491042).

Figure 3. Identification of *B2M* gene mutations in genomic DNA extracted from melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. A diagram of the *B2M* gene

structure is shown on top with indications of the identified mutation sites. Stars (*) indicate the premature stop codon in the *B2M* coding sequence from 1074MEL cells (1074stop), 1174MEL cells (1174stop) and 1106MEL, 1180MEL and 1259MEL cells (1106/1180/1259stop). The sequencing histograms encompassing the *B2M* mutation sites are shown below where the histogram marked Colo38 represents the wild-type sequence. The mutated *B2M* gene sequence shown for 1074MEL cells (1074MEL) corresponds to the anti-sense strand, whereas the mutated *B2M* gene sequence shown for 1106MEL cells (1106MEL), 1174MEL cells (1174MEL), 1180MEL cells (1180MEL), and 1259MEL cells (1259MEL) corresponds to the sense strand.

Figure 4. Restoration of HLA class I antigen expression on β_2m-transfected melanoma cell lines 1074MEL, 1106MEL and 1259MEL. Control- or IFN-γ-treated (300U/ml, 48 hrs at 37°C) clones of neo- and β_2m-transfected 1074MEL, 1106MEL, and 1259MEL cells were stained with the mAb W6/32 (opened histogram) and analyzed by flow cytometry. The isotype-matched mAb F3C25 (closed histogram) was used as a control. Results of one of three representative experiments are shown.

Figure 5. Differential *HLA-A* and *HLA-B, -C* gene product expression by the β_2m-transfected melanoma cell lines 1074MEL.β2.7, 1106MEL.β2 and 1259MEL.β2.18. (A) Higher levels of HLA-A than HLA-B, -C antigen expression by 1074MEL.β2.7 and 1259MEL.β2.18 cells and higher levels of HLA-B, -C than HLA-A antigen expression by 1106MEL.β2 cells. Cells were stained with the anti-HLA-A mAb LGIII147.41 and the anti-HLA-B, -C mAb B1.23.1 and analyzed by flow cytometry. The figure above each histogram indicates the fold increase of mean fluorescence intensity over the isotype-control background (Fold MFI). Results of one of three representative experiments are shown. (B) & (C) Higher levels of HLA-A than HLA-B, -C heavy chain expression by 1074MEL and 1259MEL cells and higher levels of HLA-B, -C than

HLA-A heavy chain expression by 1106MEL cells. (B) Control- and IFN-γ– treated (300U/ml, 48 hrs at 37°C) cells were permeabilized, stained with the mAb HCA-2 and the mAb HC-10 and analyzed by flow cytometry. Results are expressed as Fold MFI and shown in bars. (C) Lysates prepared from control- and IFN-γ– treated (300U/ml, 48 hrs at 37°C) cells were tested with the mAb HC-10 in Western blotting.

Figure 6. Selective HLA-A2 antigen loss by β_2m-transfected 1259MEL melanoma cells. Control- and IFN-γ-treated (300U/ml, 48 hrs at 37°C) β_2m-transfected 1074MEL (1074MEL.β2.7) and β_2m-transfected 1259MEL (1259MEL.β2.18) cells were stained with the mAb KS-1 and CR11-351 and analyzed by flow cytometry. The mAb Q5/13 was used to assess HLA class II antigen expression following treatment of these melanoma cells with IFN-γ. Results of one of three representative experiments are shown.

Figure 7. Selective HLA-A2 antigen loss caused by a single cytosine deletion in the *HLA-A2* gene exon 4 in 1259MEL melanoma cells. (A) Diagram of *HLA-A*0201* mRNA structure marked with the location of the primers (arrows) and of the premature stop codon (1259stop) identified in 1259MEL cells. (B) *HLA-A*0201* mRNA expression by 1259MEL cells. Total RNA was isolated from cells, reversely transcribed to first-strand cDNA, and subjected to PCR analysis with different combinations of *HLA-A-* or *HLA*0201* gene-specific primers. PCR products were run on an agarose gel and visualized by ethidium bromide staining. (C) Sequencing histogram of the single cytosine deletion found in the *HLA-A*0201* cDNA isolated from 1259MEL cells. RT-PCR-amplified *HLA-A2* exon1-exon8 (E1-E8) cDNA fragments from 1259MEL and 1074MEL cells were gel-purified and subjected to nucleotide sequencing analysis with sense primers 5pE1A2, 5pE2A and A2E3-5' or anti-sense primers AP2, 3pE3A2, A2E4-3' and 3pE8A. The histograms obtained with primer A2E3-5' are shown. Nucleotides are numbered

according to Arnett *et al.* (46) and indicated above the 1074MEL histogram. The cytosine stretch starting from nucleotide 699 is underlined. The premature stop codon TGA generated as a result of a shifted reading frame is boxed. (D) Nucleotide and deduced amino acid sequence of the partial *HLA-A2* exon 4 in 1074MEL and 1259MEL cells. The regions corresponding to codon 232 to 245 are shown. The cytosine stretches are underlined. The mis-sense codon-deduced amino acids are indicated in bold letters.

Figure 8. Three-dimensional (3-D) model of a HLA-A2-β_2m-peptide complex with a carboxyl terminus-truncated HLA-A2 heavy chain. Backbone diagram of the HLA-A2-β_2m-Hiv peptide complex (A) and the heavy chain-truncated HLA-A2-β_2m-Hiv peptide complex (B) are shown. Blue, green and red backbones indicate the HLA-A2 heavy chain, β_2m, and the Hiv peptide, respectively. The models are generated by using the RasTop 1.3.1 molecular graphics program (P. Valadon) based on the coordinates retrieved from PDB ID 1AKJ (31). The coordinates corresponding to HLA-A2 heavy chain position 1~209 (codon –24~233) were used to generate the HLA-A2-β_2m-Hiv peptide complex model with a truncated HLA-A2 heavy chain. Y209 indicates a tyrosine at position 209 (codon 233), which is the last residue before the mis-sense codon-deduced residue leucine at position 210 (codon 234) in the 1259MEL HLA-A2 heavy chain. The red-dashed cycles indicate the HLA-A2 α3 domain-β_2m contact area which is postulated to be missing in the truncated version of HLA-A2-β_2m-Hiv peptide complex.

Figure 9. Heterogeneous antigen processing machinery (APM) component expression in the melanoma cell lines 1074MEL, 1106MEL, 1174MEL, 1180MEL and 1259MEL. (A) Control- and IFN-γ-treated (300U/ml, 48hrs at 37°C) cells were permeabilized, stained with the mAb TO-2, TO-5, TO-11, TO-3, NOB-1, SY-5, SY-1, SJJ-3, HB-2, NB-1, and TO-7 and analyzed by flow cytometry. Results are expressed as Fold MFI and shown in bars. (B) Lysates prepared from

control- and IFN-γ-treated (300U/ml, 48hrs at 37°C) cells were tested with APM-specific mAb in Western blotting.

Figure 10. HLA-A2-MART-$1_{27\text{-}35}$ complex expression on 1074MEL.β2.7 melanoma cells. The neo- (1074MEL.neo) and β_2m-transfected (1074MEL.β2.7) 1074MEL cells, the neo- (1259MEL.neo) and β_2m-transfected (1259MEL.β2.18) 1259MEL cells and the β_2m-transfected 1106MEL cells (1106MEL.β2) were stained with the HLA-A2-MART-$1_{27\text{-}35}$ complex-specific scFv 8.3 tetramer (scFv 8.3 tet) and analyzed by flow cytometry. The HLA-A2, -A28-specific mAb KS-1, the HLA-A2$^+$MART-1$^+$ melanoma cells 501 and the HLA-A2$^+$MART-1$^-$ lymphoblastoid cells LG-2 were used as controls.

Fig. 1

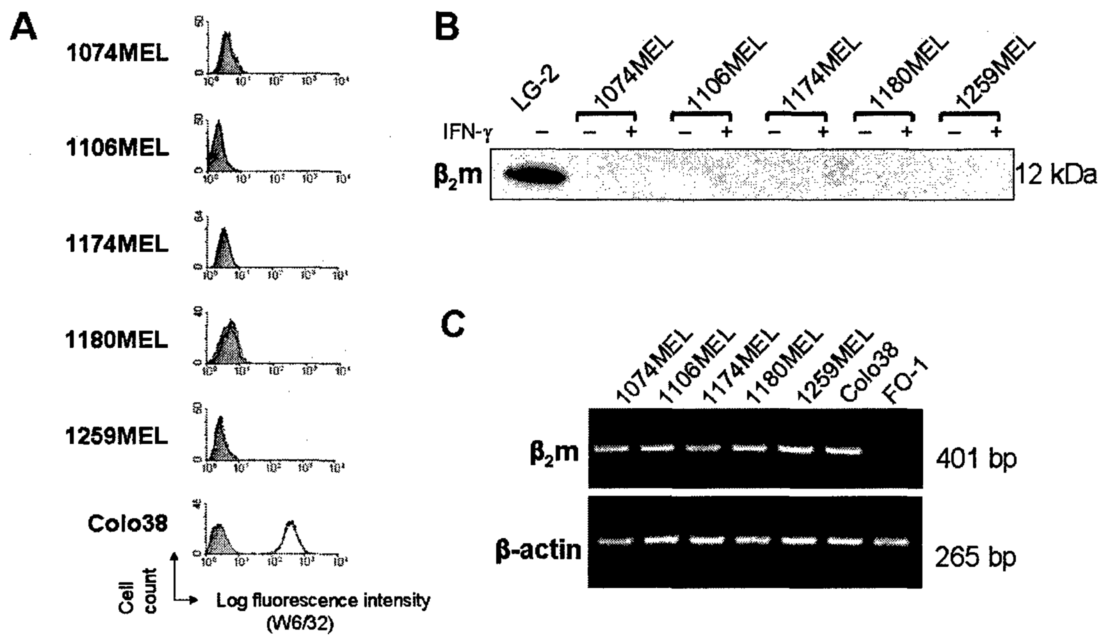
A
1074MEL
1106MEL
1174MEL
1180MEL
1259MEL
Colo38
Cell count
Log fluorescence intensity (W6/32)
B
LG-2
1074MEL
1106MEL
1174MEL
1180MEL
1259MEL
IFN-γ
β2m
12 kDa
C
1074MEL
1106MEL
1174MEL
1180MEL
1259MEL
Colo38
FO-1
β2m
401 bp
β-actin
265 bp

Fig. 2

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                                                                                         ▼
Consensus ATGTCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAAGATTCAGGTTTAC   exon 1/exon 2
1074MEL   --A---------------------------------------------------------------------------------------
1106MEL   --------------------------------------^^--------------------------------------------------
1174MEL   ------------------------------------------------------------------------------------------
1180MEL   --------------------------------------^^--------------------------------------------------
1259MEL   --------------------------------------^^--------------------------------------------------
Consensus M S R S V A L A V L A L L S L S G L E A I Q R T P K I Q V Y  30
1074MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1106MEL   - - - - - - - - - - - - -   S F W P G G Y P A Y S K D S G L
1174MEL   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
1180MEL   - - - - - - - - - - - - -   S F W P G G Y P A Y S K D S G L
1259MEL   - - - - - - - - - - - - -   S F W P G G Y P A Y S K D S G L

Consensus TCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCCTGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTGAAGTTGACTTACTG
1074MEL   ------------------------------------------------------------------------------------------
1106MEL   ------------------------------------------------------------------------------------------
1174MEL   -G----------------------------------------------------------------------------------------
1180MEL   ------------------------------------------------------------------------------------------
1259MEL   ------------------------------------------------------------------------------------------
Consensus S R H P A E N G K S N F L N C Y V S G F H P S D I E V D L L  60
1074MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1106MEL   L T S S S R E W K V K F P E L L C V W V S S I R H * . . . .
1174MEL   * . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1180MEL   L T S S S R E W K V K F P E L L C V W V S S I R H * . . . .
1259MEL   L T S S S R E W K V K F P E L L C V W V S S I R H * . . . .

Consensus AAGAATGGAGAGAGAATTGAAAAAGTGGAGCATTCAGACTTGTCTTTCAGCAAGGACTGGTCTTTCTATCTCTTGTACTACACTGAATTC
1074MEL   ------------------------------------------------------------------------------------------
1106MEL   ------------------------------------------------------------------------------------------
1174MEL   ------------------------------------------------------------------------------------------
1180MEL   ------------------------------------------------------------------------------------------
1259MEL   ------------------------------------------------------------------------------------------
Consensus K N G E R I E K V E H S D L S F S K D W S F Y L L Y Y T E F  90
1074MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1106MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1174MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1180MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1259MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                                                                                 ▼
Consensus ACCCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCATGTGACTTTGTCACAGCCCAAGATAGTTAAGTGGGATCGAGACATGTAA   exon 2/exon 3
1074MEL   ------------------------------------------------------------------------------------------
1106MEL   ------------------------------------------------------------------------------------------
1174MEL   ------------------------------------------------------------------------------------------
1180MEL   ------------------------------------------------------------------------------------------
1259MEL   ------------------------------------------------------------------------------------------
Consensus T P T E K D E Y A C R V N H V T L S Q P K I V K W D R D M *  120
1074MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1106MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1174MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1180MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
1259MEL   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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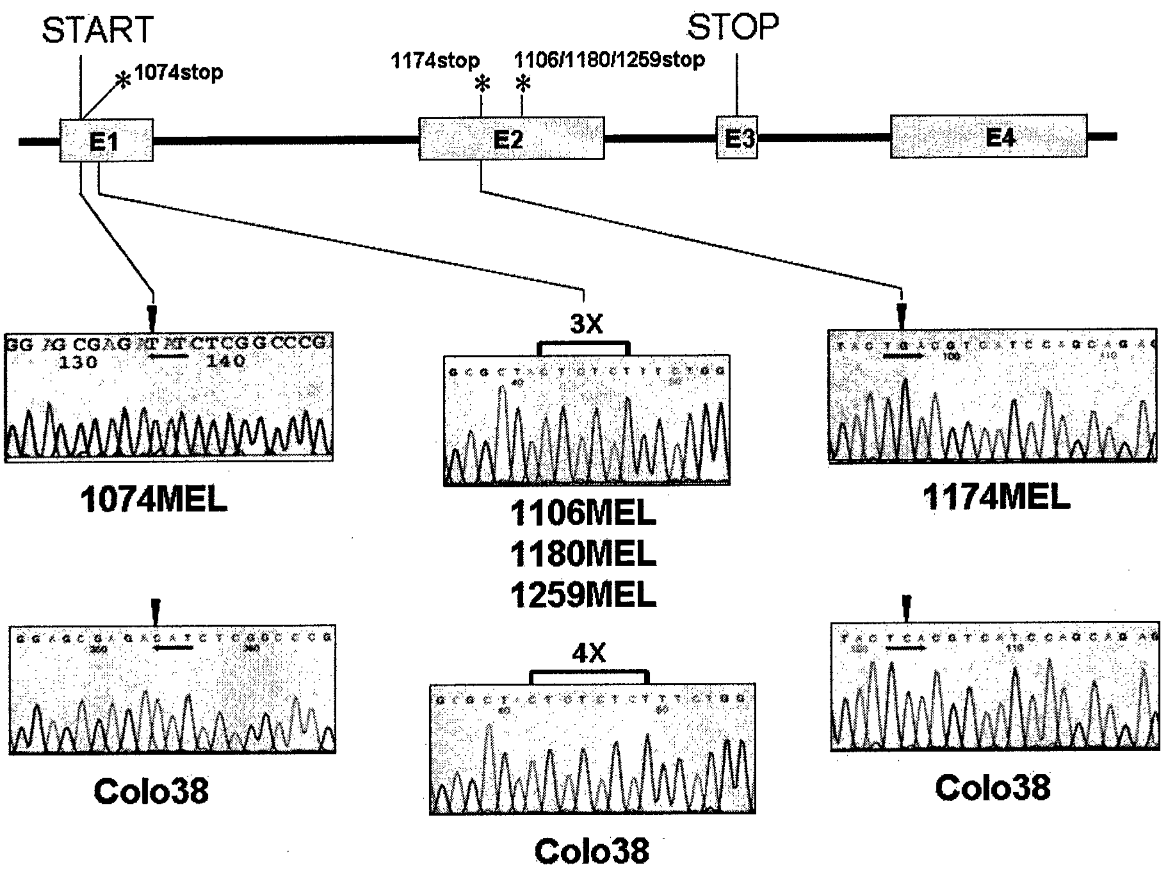
Fig. 3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1174stop
1106/1180/1259stop
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E1
E2
E3
E4
3X
4X
1074MEL
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1174MEL
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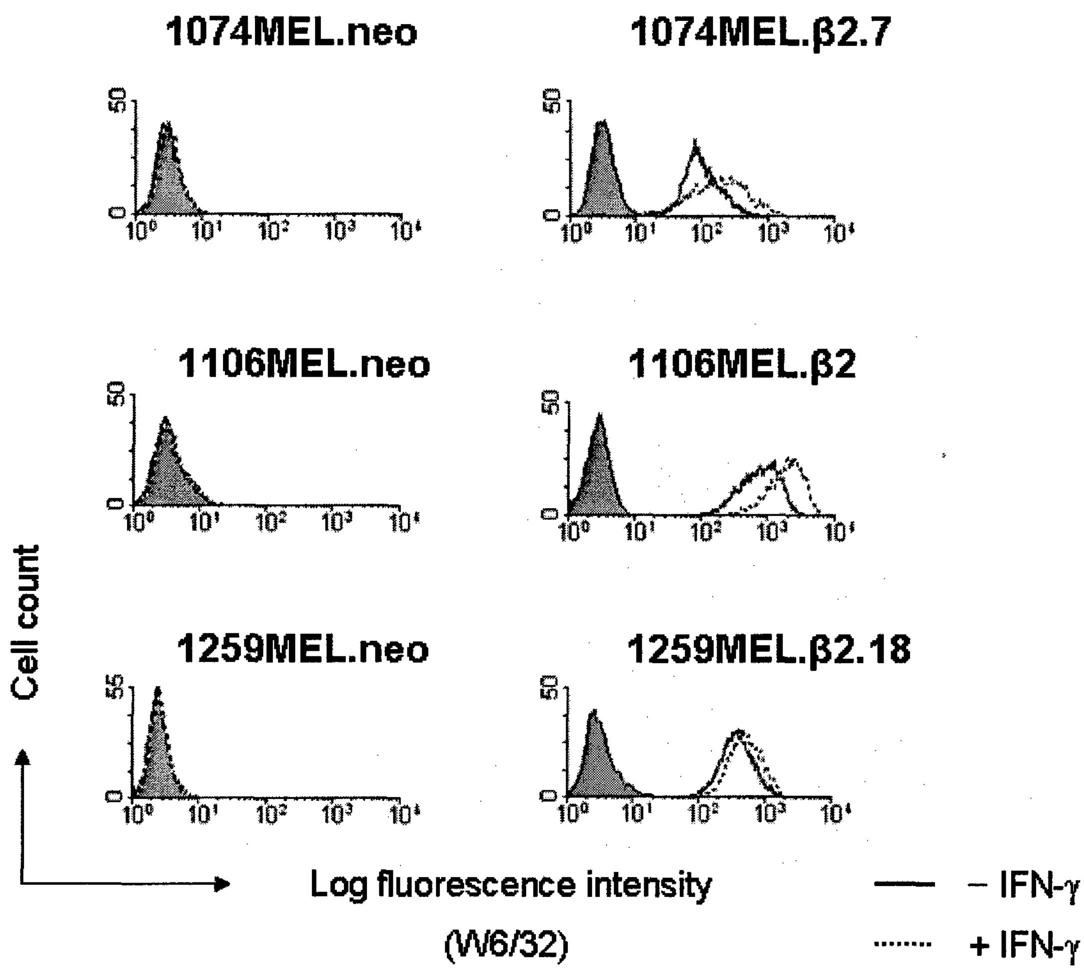
Colo38
Colo38

Fig. 4



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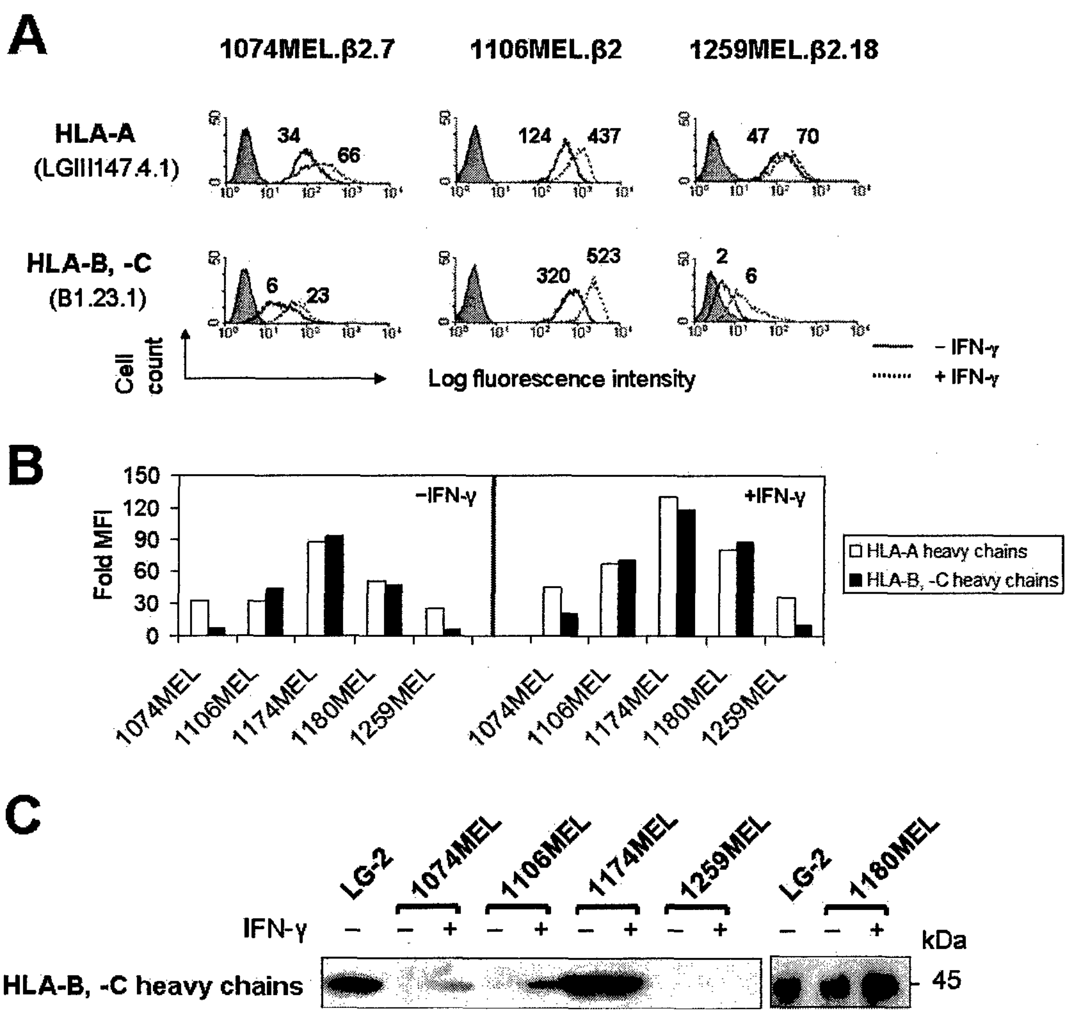
1106MEL.neo
1106MEL.β2
1259MEL.neo
1259MEL.β2.18
50
55
0
10⁰ 10¹ 10² 10³ 10⁴
Cell count
Log fluorescence intensity
(W6/32)
– IFN-γ
+ IFN-γ

Fig. 5


A
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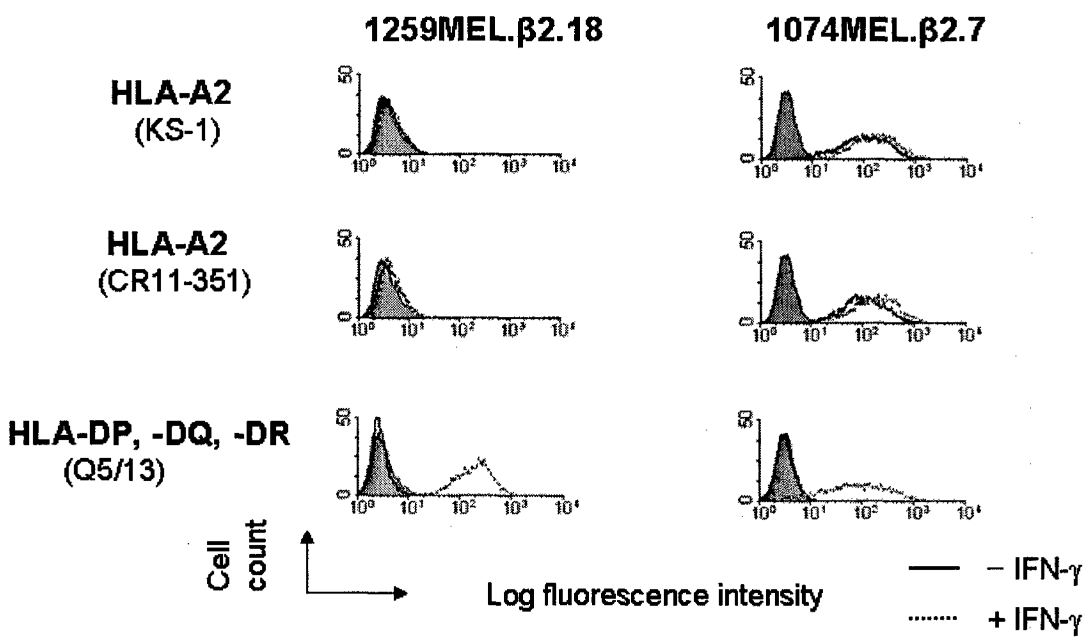
1106MEL.β2
1259MEL.β2.18
HLA-A
(LGIII147.4.1)
34
66
124
437
47
70
HLA-B, -C
(B1.23.1)
6
23
320
523
2
6
Cell count
Log fluorescence intensity
– IFN-γ
+ IFN-γ
B
Fold MFI
150
120
90
60
30
0
–IFN-γ
+IFN-γ
HLA-A heavy chains
HLA-B, -C heavy chains
1074MEL
1106MEL
1174MEL
1180MEL
1259MEL
C
LG-2
1074MEL
1106MEL
1174MEL
1259MEL
LG-2
1180MEL
IFN-γ
kDa
HLA-B, -C heavy chains
45

Fig. 6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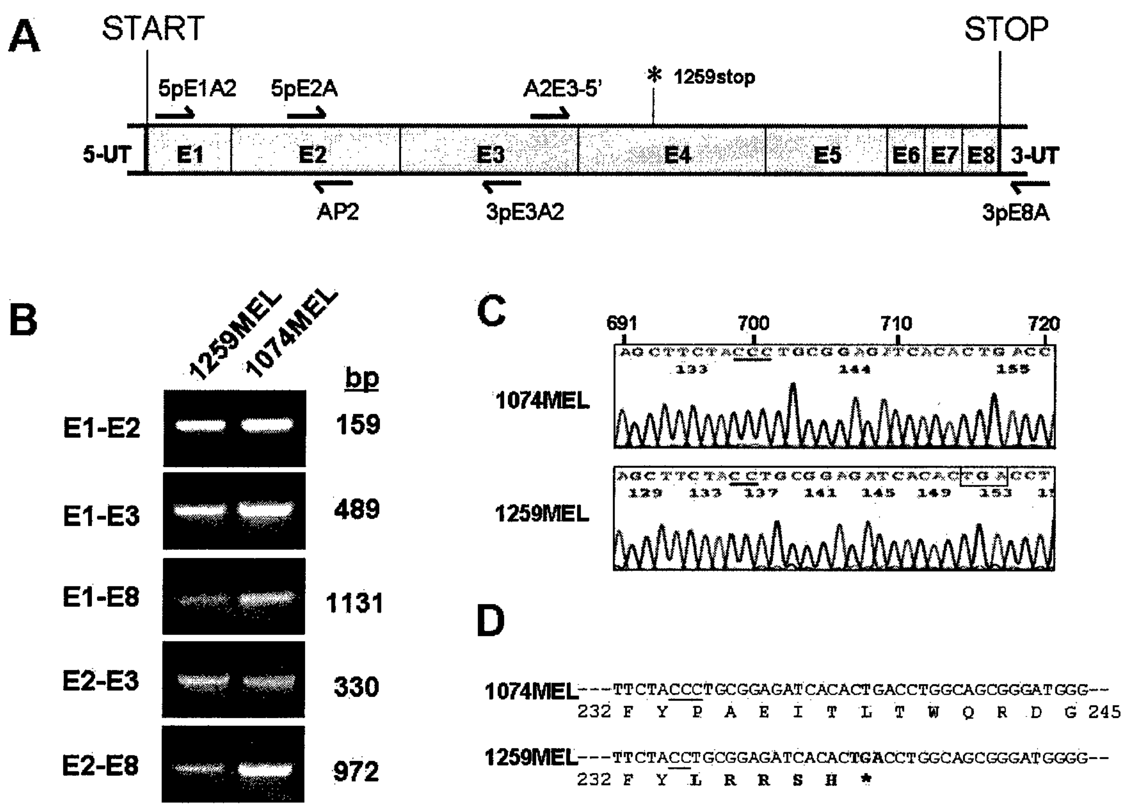
1074MEL.β2.7
HLA-A2
(KS-1)
HLA-A2
(CR11-351)
HLA-DP, -DQ, -DR
(Q5/13)
Cell count
Log fluorescence intensity
– IFN-γ
+ IFN-γ

Fig. 7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START
STOP
5pE1A2
5pE2A
A2E3-5'
* 1259stop
5-UT
E1
E2
E3
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E5
E6
E7
E8
3-UT
AP2
3pE3A2
3pE8A
B
1259MEL
1074MEL
bp
E1-E2
159
E1-E3
489
E1-E8
1131
E2-E3
330
E2-E8
972
C
691
700
710
720
1074MEL
1259MEL
D
1074MEL---TTCTACCCTGCGGAGATCACACTGACCTGGCAGCGGGATGGG--
232 F Y P A E I T L T W Q R D G 245
1259MEL---TTCTACCTGCGGAGATCACACTGACCTGGCAGCGGGATGGGG--
232 F Y L R R S H *

Fig. 8

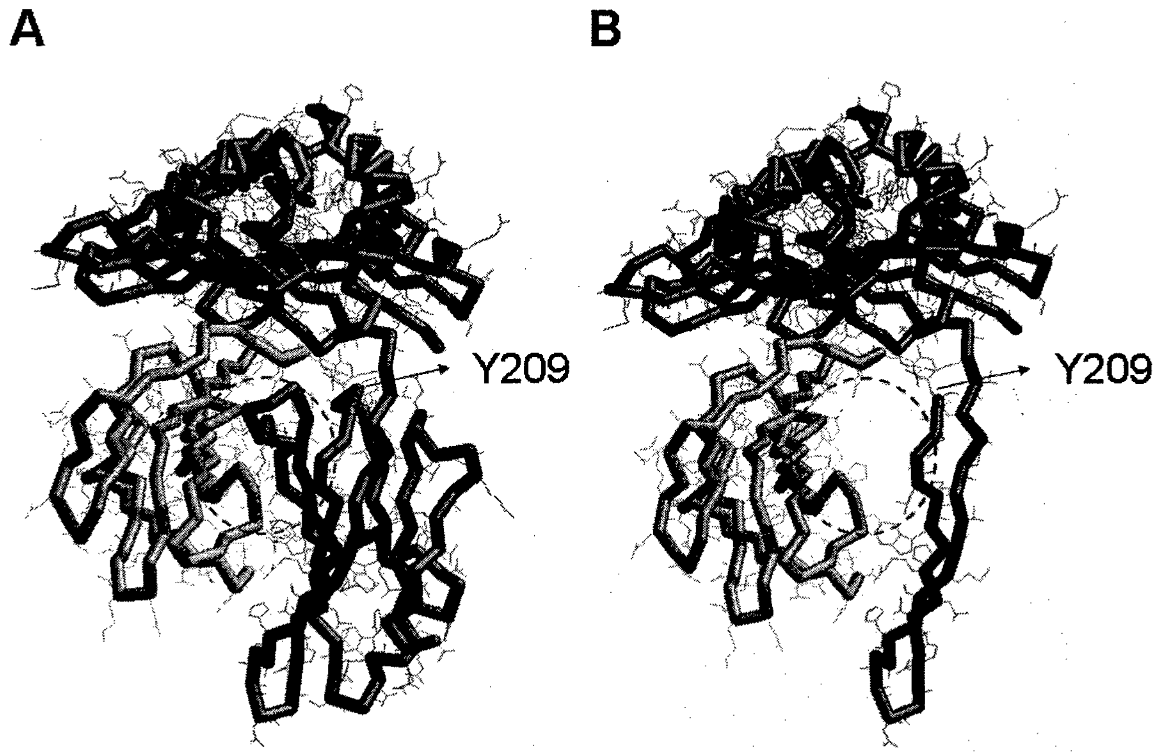
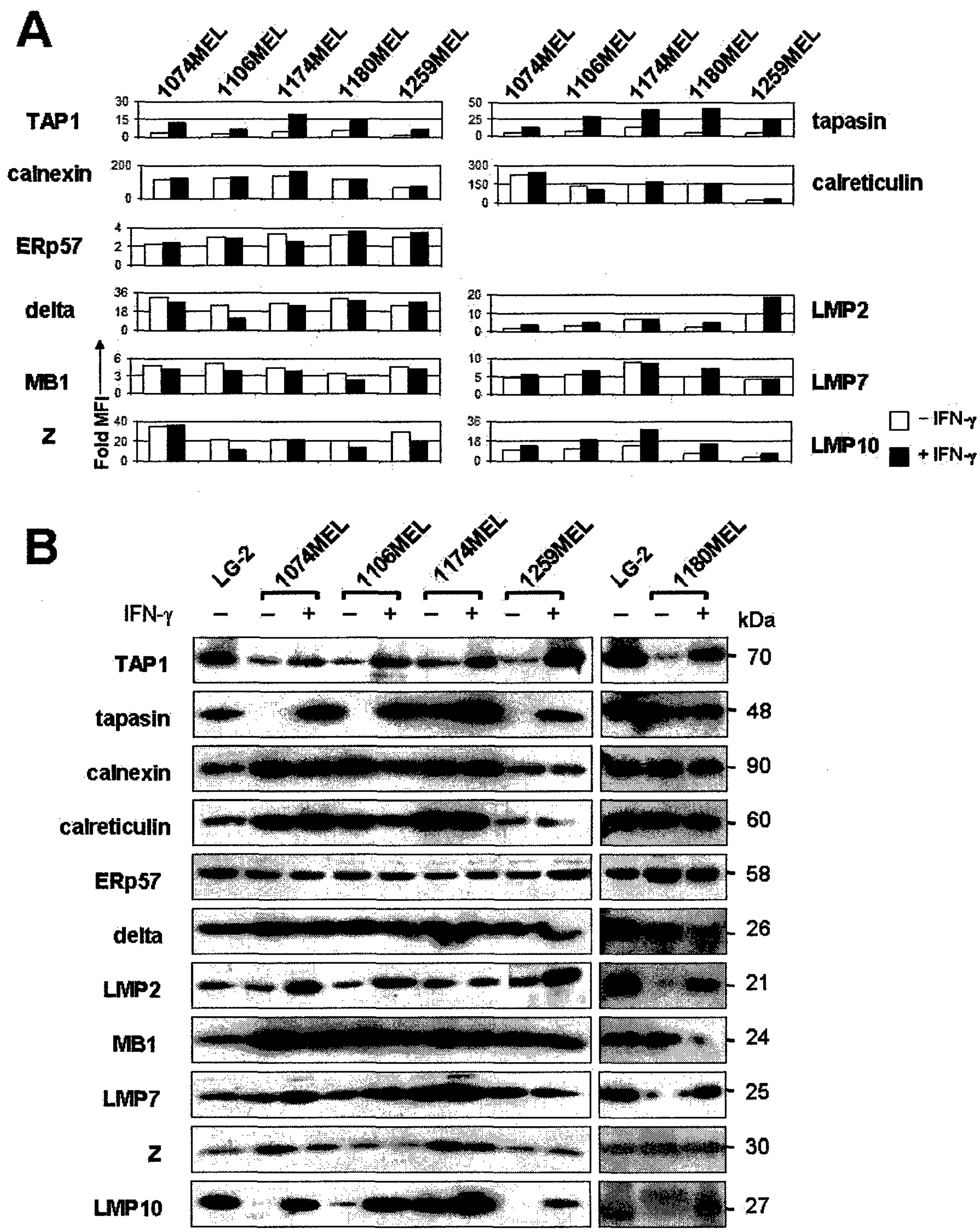


Fig. 9


A
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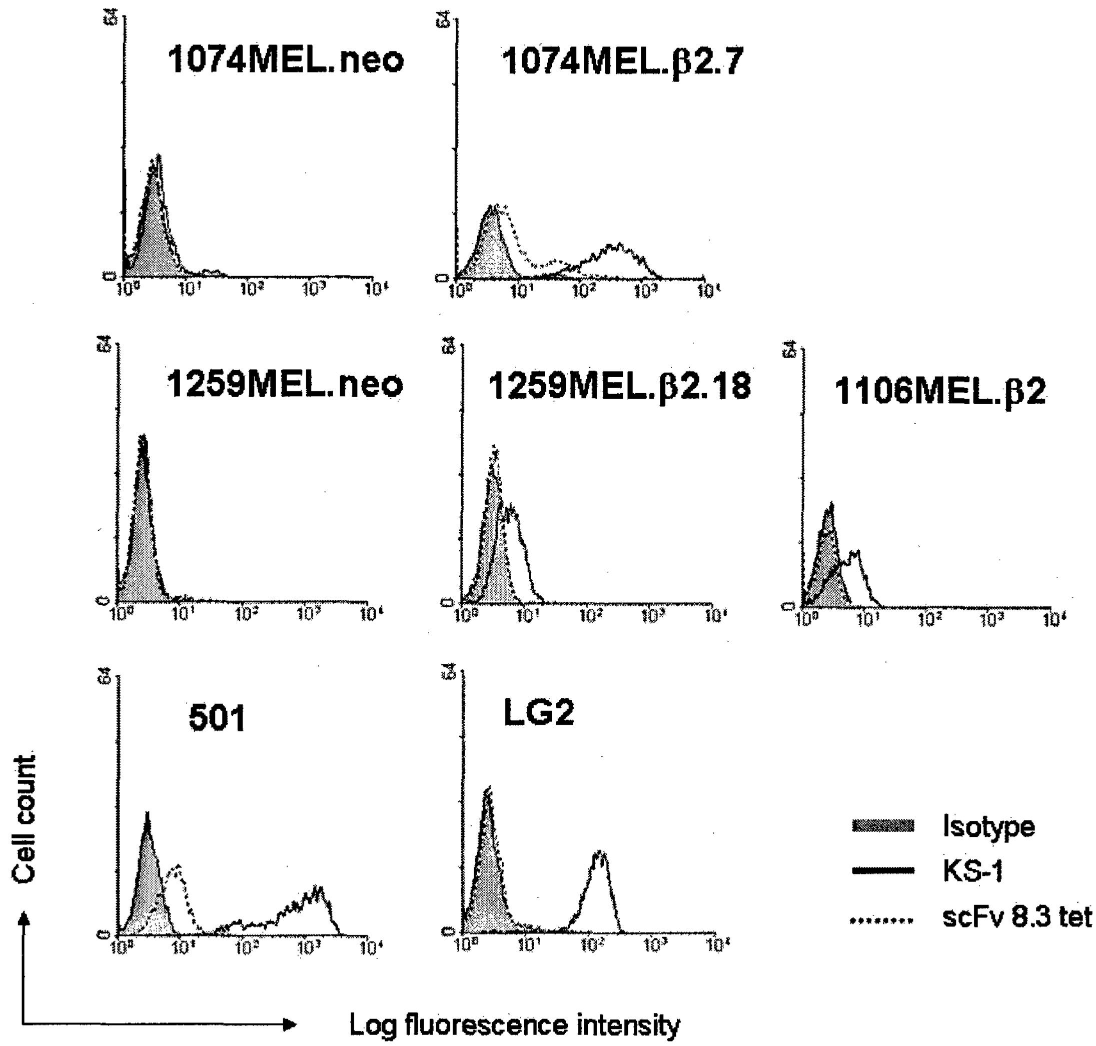
1106MEL
1174MEL
1180MEL
1259MEL
TAP1
tapasin
calnexin
calreticulin
ERp57
delta
LMP2
MB1
LMP7
Z
LMP10
Fold MFI
− IFN-γ
+ IFN-γ
B
LG-2
IFN-γ
kDa
70
48
90
60
58
26
21
24
25
30
27

Fig. 10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1074MEL.β2.7
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1259MEL.β2.18
1106MEL.β2
501
LG2
Cell count
Log fluorescence intensity
Isotype
KS-1
scFv 8.3 tet

HLA antigen changes in malignant tumors of mammary epithelial origin: molecular mechanisms and clinical implications.

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Running Title: HLA antigen changes in breast carcinoma cells

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Introduction

As in other types of malignant tumors in humans and in other animal species, malignant transformation of human mammary epithelial cells may be associated with changes in gene expression and in their antigenic profile. Among the latter, loss or downregulation of classical HLA class I antigens [Fig. 1A] and induction of non-classical HLA class I antigens [Fig. 1B] as well as of HLA class II antigens [Fig. 1C] are of particular interest to tumor immunologists and clinical oncologists because of the critical role these antigens play in the generation of tumor antigen (TA)-specific immune responses [1,2], as well as their ability to modulate the interactions of NK cells [3] and T cell subpopulations [4,5] with target cells [Fig 1A.}. In this chapter we will first discuss the available information about the frequency of abnormalities in classical HLA class I antigen expression in breast carcinoma lesions and the potential underlying molecular defects. Second, we will address the frequency of the non-classical HLA class I molecules HLA-E, F, and G, as well as, the natural killer (NK) cell activating ligands MICA/B and ULBP antigen expression in breast carcinoma lesions. Third, we will address the functional relevance and potential clinical significance of abnormalities in classical HLA class I antigen, non-classical HLA class I antigen and NK cell activating ligand expression in breast carcinoma lesions. Lastly, we will discuss the frequency of HLA class II antigen expression in breast carcinoma lesions, the potential underlying molecular mechanisms affecting HLA class II antigen expression and the potential clinical significance of HLA class II antigen expression in breast carcinoma lesions.

I. Detection of HLA antigens in breast carcinoma lesions

Analysis of HLA antigen expression in breast carcinoma lesions has primarily been performed through immunohistochemical (IHC) staining of surgically removed breast carcinoma lesions with monoclonal antibodies (mAb). The latter have been utilized because the limited specificity and high background staining obtained with conventional allo- and xeno-antisera have hindered their application in IHC techniques. Most of the studies have made use of mAb recognizing monomorphic determinants of HLA antigens, since for many years there has been a limited availability of mAb recognizing HLA class I and class II allospecificities. Only in recent years mAb to a large number of allospecificities have become available due to the major effort to develop mAb for HLA typing. Unfortunately, most of the available mAb to HLA allospecificities do not work in IHC reactions. As a result, the expression of only a limited number of HLA class I and class II allospecificities has been investigated in malignant lesions. These limitations are not likely to be overcome in the near future because the declining interest in the use of antibody-based methods for HLA typing has had a negative impact on the development of mAb recognizing HLA class I and class II alleles.

A majority of the published studies have utilized surgically removed frozen tissue sections as substrates in IHC staining, since the determinants recognized by most available mAb are not expressed in formalin-fixed, paraffin-embedded tissues. The use of frozen tissue introduces the least technical fixation artifact and is a practical option to preserve antigens that may be lost during standard formalin-fixation [6-8]. Analysis of frozen tissue sections has provided useful information regarding the tissue distribution of HLA antigens, however frozen tissues do not represent the most optimal substrate in IHC staining for a number reasons which account for the use of formalin-fixed, paraffin-embedded tissues as substrates in IHC staining in routine IHC laboratories in departments of pathology. First, cyropreservation of tissue may result in loss of structural and cellular detail rendering tissue unacceptable for morphologic evaluation and IHC analysis [6-8]. Second, frozen tissue requires special storage in a liquid nitrogen freezer and has a life span of about 2 years before desiccation renders the tissue unsuitable for IHC technique [6-8]. Third, handling of frozen tissue requires significantly more time than routine formalin-fixation and large pieces of tissue cannot be processed [6-8]. Fourth, repeated freeze/thaw cycles may alter antigen expression over time such that antigens initially expressed may be lost or their expression diminished [6-8]. Lastly, the use of frozen tissue hampers retrospective studies, since most archived clinical samples are fixed with formalin and embedded with paraffin [6-8]. These limitations have provided a stimulus to look for HLA antigen-specific mAb which stain formalin-fixed, paraffin-embedded tissues. In recent years, mAb that detect monomorphic determinants of HLA class I and class II antigens in formalin-fixed, paraffin-embedded tissues have been developed [9]. Furthermore, in our laboratory we have recently developed a HLA-A-specific mAb, which stains formalin-fixed, paraffin-embedded tissues (Cho *et al.* manuscript in preparation). These reagents, along with improvements in methods for antigen retrieval [6-8], have allowed the use of formalin-fixed, paraffin-embedded tissues to analyze HLA class I and class II antigen expression in surgically removed tumors, thus facilitating the use of archived clinical samples in retrospective studies. It is hoped that the availability of this methodological improvement will facilitate the analysis of malignant lesions for HLA antigen expression in departments of pathology. However, it should be stressed that frozen tissue sections must still be used as substrates in IHC staining in order to characterize HLA allospecificity expression. This is due to the fact that the polymorphic determinants that define each allospecificity are conformational in nature and are lost during the fixation of tissues with formalin and their embedding with paraffin.

Analysis of IHC staining relies on the microscopic reading of surgically removed tissue sections that have been stained with mAb in immunoperoxidase reactions. Evaluation of the results is largely subjective and relies on the assessment of tissue staining as estimated by independent observers. Results are often expressed as the percentage of malignant cells stained in a tissue section and level of staining intensity is recorded. Although IHC technique has proven to be very valuable to analyze HLA antigen expression in surgically removed tumors, the results reported in the literature have to be interpreted with caution for several reasons. First, the subjective nature of the evaluation of IHC staining of tissue sections in conjunction with the lack of standardized criteria to score the staining results have led to a marked variation in the reported frequency of HLA changes in breast carcinoma lesions. At present, we do not know whether these differences are technical or biological in nature. Second, there is no information regarding the reproducibility of IHC staining results among different laboratories, although there is some data which demonstrates that variations in the percentage of stained cells enumerated by two experienced observers within the same laboratory is less than 10% [10]. Third, staining intensity of adjacent normal structures (i.e. lymphoid and endothelial cells) is often used as an internal control to evaluate the staining intensity of malignant cells. However, endothelial and lymphoid cells do not represent the most appropriate control for evaluating HLA antigen expression by malignant cells, since in most cases the tumor cells being evaluated are of a different lineage. Lastly, it is noteworthy that the functional significance of the results of IHC staining remains to be determined, since it is not known whether the lack of staining of tumor cells by anti-HLA class I antigen mAb in IHC reactions is a reliable predictor of tumor cell resistance to TA-specific cytotoxic T lymphocyte (CTL) mediated lysis. In this regard, no study has compared the sensitivity of antibody-based and CTL-based assays to detect HLA class I antigens on the cell membrane.

In an attempt to overcome some of the aforementioned limitations and standardize the evaluation of HLA antigen expression in malignant lesions, the HLA and Cancer component of the 12th International Histocompatibility Workshop established an international classification system for the assessment of HLA antigen expression in malignant lesions [11]. According to this classification system, lesions are scored as positive, heterogeneous and negative, when the percentage of stained tumor cells in the entire lesion is more than 75%, between 75% and 25% inclusive, and less than 25%, respectively. Furthermore, staining intensity is scored as (absent), $\pm$ (dull) and + (bright) and staining intensity of adjacent normal structures (i.e. lymphoid and endothelial cells) are used as an internal control to evaluate staining intensity of malignant cells. Nevertheless, it should be noted that evaluation of IHC staining of surgically removed lesions remains largely subjective and still suffers from a lack of appropriate controls. These limitations hinder the quantitative assessment of HLA expression in breast carcinoma lesions and make it difficult to assess the differentiation between HLA class I antigen loss and marked downregulation. It is hoped that this limitation will be overcome in the near future by the development of equipment for computer-based reading of IHC staining. These improvements should facilitate the standardization of the analysis of HLA antigen expression in malignant lesions and should provide more quantitative data about HLA antigen expression in both normal and pathological tissues.

II. Frequency and molecular mechanisms underlying abnormal HLA class I antigen phenotypes in breast carcinoma lesions.

To date, approximately 2000 surgically removed breast carcinoma lesions have been analyzed for classical HLA class I antigen expression [9, 12-41]. Most of the studies performed have only utilized mAb W6/32, which recognizes a monomorphic determinant on HLA class I antigens [42], in IHC staining of surgically removed breast carcinoma lesions. The frequency of HLA class I antigen abnormalities in breast carcinoma lesions has been found to average around 50%, although frequencies have been noted to vary between 0% (0/63 lesions) [34] and 90% (158/197 lesions) [38]. It is likely that these differences partly reflect the subjective evaluation of IHC staining and/or the system used to score HLA class I antigen expression. However, we cannot exclude the possibility that the variable frequency of HLA class I antigen abnormalities described in the literature is due to differences in characteristics of the patient population and/or type of breast carcinoma analyzed, e.g. fibroadenoma, ductal carcinoma, etc., since the role of this variable has not been investigated. On the other hand, differences in the sensitivity of IHC staining caused by the characteristics of the mAb used are not likely to be responsible for variable frequency of HLA class I antigen abnormalities described in the literature, since more than 80% of the studies performed have utilized mAb W6/32. In this regard, lack of staining by mAb W6/32 is likely to represent loss of HLA class I antigens and not only of the corresponding determinant, since information derived from the analysis of 25 frozen breast carcinoma lesions with RT-PCR, western blot or IHC staining with mAb W6/32 found the frequency of HLA class I antigen abnormalities to be nearly identical [39,40].

As in other types of tumors [43] distinct defects in HLA class I antigen expression have been identified in breast carcinoma lesions. They include (i) total loss and/or downregulation of the gene products of HLA-A, B and C loci; (ii) loss of one HLA class I antigen haplotype; (iii) selective loss and/or downregulation of the gene products of one HLA class I locus; and (iv) selective loss of one HLA class I allospecificity [Fig. 2]. However, at variance with other types of tumors there is little to no information regarding the molecular mechanism(s) underlying the HLA class I antigen abnormalities identified in breast carcinoma lesions. The scanty information reflects, at least in part, the paucity of breast carcinoma cell lines with demonstrable HLA class I antigen defects, therefore hindering the characterization of the underlying molecular mechanism(s). For this reason, potential mechanisms underlying HLA class I antigen abnormalities in breast carcinoma lesions will be presented in the context of those mechanisms that have been identified in other types of malignancies.

i. Total HLA class I antigen loss and marked downregulation

For the reasons reviewed before it is very difficult to differentiate between total HLA class I antigen loss and marked downregulation. Therefore, we have grouped the two types of defects. The frequency of HLA class I antigen loss and/or downregulation in breast carcinoma lesions ranges between 20%-50%, among most of the reported studies, although frequencies have been reported as high as 100% (11/11 lesions) [17] and as low as 0% (0/53 lesions) [22]. The frequency of total HLA class I antigen loss and/or downregulation in breast carcinoma lesions is higher than that found in cutaneous melanoma lesions and similar to that found in prostate cancer lesions [9] [Fig 3]. These differences are likely to reflect the time length between onset of tumor and diagnosis, since a long interval gives tumor cells more chances to acquire mutations in the genes involved in HLA class I antigen expression, thus allowing mutated cells to over-grow those cells without abnormalities in their HLA class I phenotype in the presence of

T cell selective pressure. Studies with other types of tumors [9] have shown that total HLA class I antigen loss and/or downregulation in malignant cells may be caused by defects in β_2-microglobulin (β_2m) synthesis, epigenetic alterations involving the HLA class I heavy chain loci, defects in the regulatory mechanisms that control HLA class I antigen expression and/or abnormalities in one or more of the antigen processing machinery components.

i.1. Defects in $\beta_2 m$ expression

Defects in β_2m, which is required for the formation of the HLA class I heavy chain-β_2m-peptide complex and its transport to the cell membrane, caused by loss of one copy of the *$\beta_2 m$* gene located on chromosome 15 [45] and by structural mutations in the remaining *$\beta_2 m$* gene [44], which in most cases inhibit translation and in a few cases inhibit transcription. Analysis of about 856 surgically removed breast carcinoma lesions using IHC staining with anti-human β_2m mAb GRH-1, BBM.1 L368 and NAMB-1 as well as with rabbit polyclonal anti-human β_2m antibodies has demonstrated that the frequency of β_2m loss ranges between 4% (3/77 lesions) [24] and 67% (36/53 lesions) [13], with an average of 45% among all reported studies [13-15,18,20,22,24,26,28,31,34-37,38-40]. As for the mechanisms underlying this lack of detection of β_2m, to the best of our knowledge, no *$\beta_2 m$* gene mutations have been identified in the analysis of about 150 breast carcinoma lesions [34,40]. One study has analyzed loss of heterozygosity (LOH) at chromosome 15 in 99 surgically removed breast carcinoma lesions and found it to be present in 28 (29%) of the lesions [46]. However, it is not clear whether the remaining copy of the *$\beta_2 m$* gene harbored any mutations or what effect LOH at chromosome 15 had on HLA class I antigen expression, since neither was analyzed. Therefore, unlike some malignancies, in which *$\beta_2 m$* gene mutations have been frequently identified (e.g. cutaneous melanoma [9]), structural defects in the β_2m gene appear to be a rare event in breast carcinoma cells. These findings do not appear to be unique for breast carcinoma, since β_2m gene mutations have not been identified in head and neck squamous cell carcinoma (HNSCC), colorectal, renal and bladder carcinoma lesions with total HLA class I antigen loss [47-51]. Taken together, these observations suggest that mutations in the β_2m gene may not be the predominant molecular mechanism underlying total HLA class I antigen loss in breast carcinoma lesions. Alternative mechanisms underlying β_2m loss, include abnormal post-transcriptional regulation of β2m, which has been recently described in a drug resistant breast carcinoma cell line [52].

i.2. Epigenetic mechanisms

Epigenetic mechanisms such as hypermethylation of the HLA-A, B and C gene promoter regions and/or altered chromatin structure of the HLA class I heavy chain gene promoters [50,55,56] have also been found to underlie total HLA class I antigen loss and/or downregulation. This phenomenon has been implicated as a major mechanism for transcriptional inactivation of HLA class I antigen genes in esophageal squamous cell and oesophageal carcinomas [55] and is also responsible for total HLA class I antigen loss in cutaneous melanoma [56]. Although epigenetic alterations of the HLA class I antigen loci have not been described in breast carcinoma cells, this mechanism is likely to play a role since a large body of evidence has demonstrated that CpG island hypermethylation and/or altered chromatin acetylation patterns are implicated in loss of expression of a variety of critical genes in breast carcinoma cells [57,58]. Therefore, characterization of epigenetic changes in HLA class I antigen downregulation which is frequently found in breast carcinoma lesions appears to be a fruitful area of investigation.

i.3. Defects in HLA class I antigen gene regulation and/or antigen processing machinery component expression

Convincing, although limited, evidence suggests that HLA class I antigen expression in breast carcinoma cell lines can be restored and/or enhanced by *in vitro* incubation with IFN-γ, IFN-α, IL-1, TNF-α and hormones such as 17-β-estradiol [59-62], suggesting that altered regulation of non-mutated genes such as the antigen processing machinery (APMC) or DNA binding factors may play a part in defects in HLA class I antigen expression. In the latter case, transcriptional activity of HLA class I heavy chain genes has been to shown be suppressed by the presence of a silencer located at the distal promoter [53,54], however the role of this silencer in total HLA class I antigen loss and/or downregulation in breast carcinoma cells has yet to be investigated. More recently, a 9 base pair negative *cis*-regulatory element (NRE) has been identified in the 5' flanking region of HLA class I genes and has been shown to act as an inhibitor of the HLA enhancer element in breast carcinoma cell lines [61]. Interestingly, different DNA-binding factors have been shown to preferentially bind to this element in the breast carcinoma cell lines MCF-7 and T-47D, which express markedly lower HLA class I antigen levels than a cell line derived from normal breast epithelial cells. The exact functional role of NRE in relationship to HLA class I antigen gene regulation *in vivo* remains to be determined.

The APMC play a crucial role in the assembly of functional HLA class I antigen-peptide complexes [63] [Fig. 4] and defects in APMC expression can result in total HLA class I antigen loss and/or downregulation [9,44]. To date, there is limited information in the literature about APMC expression in breast carcinoma lesions. In addition, it is noteworthy that there is no information concerning APMC expression in normal mammary epithelial cells. Consequently, there is no information as to what constitutes normal or pathological expression profiles of these components. In this regard, many of the reported studies have utilized inappropriate controls, i.e. lymphoid cell lines, to determine the relative amount of APMC expression in breast carcinoma cell lines. Therefore, it is not clear whether the 'abnormalities' that have been reported in the literature reflect actual APMC defects or merely normal variation in APMC expression among cell lines of different embryological origin. The paucity of the available information reflects the limited or lack of availability of antibodies to quantitate APMC expression. This limitation has been overcome by the recent development of antibodies [64] which in conjunction with newly developed methodology [65] can provide quantitative information about intracellular APMC expression. Representative examples are shown in Fig. 5. Due to the aforementioned limitations, most of the studies performed have utilized reverse transcription-polymerase chain reaction (RT-PCR) analysis in order to assess APMC expression in breast carcinoma cell lines [39,40,62]. Although these studies are conclusive when mRNA is not detected, they do not provide any information about the level and/or function of the proteins expressed when mRNA is expressed given the lack of close correlation between the level of transcription and translation in malignant cells. Hence, the results of these studies should be interpreted with caution.

The generation of HLA class I antigen-TAA derived peptide complexes begins with the cleavage of intracellular proteins into peptides by the 26S proteasome [63]. The activity of the proteasome can be modulated by IFN-γ, which induces the expression of the proteasome activator 28 (PA28), also known as the 11S cap, and the exchange of the three constitutive active sites $\beta 1$, $\beta 2$ and $\beta 5$ with the immunosubunits LMP2, LMP10 (MECL-1) and LMP7, respectively, creating the immunoproteasome [63]. PA28 and immunosubunit (LMP) expression favors the generation of antigenic peptides demonstrating increased binding affinity for HLA class I antigens and enhances the recognition of target cells by antigen-specific CTL. However, it should be noted that some peptides, primarily of self-origin, are not processed by the immunoproteasome [66] and expression of these immunosubunits is not essential for overall antigen presentation [63]. It is generally assumed that immunosubunit expression is not constitutive within a cell, but induced upon exposure to cytokines i.e. IFN-γ, thereby increasing the number of peptides capable of binding HLA class I antigens. However, at variance with this notion, basal expression of LMP2, LMP7 and LMP10 has been observed in mouse and in human normal cells of different histology in the absence of IFN-γ [63,67,68]. Therefore, caution should be exercised when one interprets studies which describe LMP immunosubunit expression as 'downregulated' or 'abnormal', since the phenotype of the normal counterparts is not known in many cases.

Thus far, 22 surgically removed breast carcinoma lesions and 20 human breast carcinoma cell lines have been analyzed for LMP2, LMP7 and LMP10 (MECL-1) expression [62,69,70]. Using RT-PCR, the level of LMP2, LMP7 and LMP10 expression when compared to a T/B cell hybrid line has been shown to be 'downregulated' in 34%, 20% and 34% of the 20 analyzed cell lines, respectively. Combined deficiencies in LMP2, LMP7 and LMP10 expression were observed in 8 of the 20 cell lines studied. These 'deficits' are likely to be caused by regulatory mechanisms, since they could be corrected by administration of IFN-γ. Interestingly, a correlation was found between LMP2 and HLA class I antigen downregulation in breast carcinoma cell lines. However, analysis of 22 surgically removed formalin-fixed, paraffin-embedded breast carcinoma lesions found 100% of the lesions to express LMP2 and LMP7 with variable staining intensity and no association between LMP2 or LMP7 expression and tumor grading [70], raising the possibility that the *in vitro* finding is an artifact of *in vitro* cell culture.

The functional significance of impaired LMP2, LMP7 and LMP10 expression in breast carcinoma cells remains to be determined. However the role these catalytic subunits play in the generation of TAA derived peptides suggests that these variations may lead to alterations in the repertoire of peptides presented on HLA class I antigens by malignant cells.

The restoration of HLA class I antigen expression in malignant breast carcinoma cells by IFN-γ suggests that, of the APMC, defects in TAP1 and/or TAP2 expression may also play a part in abnormal HLA class I antigen expression. The latter are responsible for the quantitative and qualitative translocation of peptides into the endoplasmic reticulum [63]. To date, TAP1 and TAP2 expression has been investigated in 20 breast carcinoma cell lines [62,69] and in 141 surgically removed primary breast carcinoma lesions [39,40,70,71]. Using semi-quantitative RT-PCR, TAP1 and TAP2 were found to be downregulated in 19 of 20 and 18 of 20 cell lines, respectively. TAP1 and TAP2 expression was restored by IFN-γ. Therefore these abnormalities are likely to be caused by regulatory and not structural defects, although the functional characteristics of the induced proteins were not determined. Three studies have investigated TAP expression in surgically removed breast carcinoma lesions. The frequency of TAP1 and TAP2 downregulation in the 141 breast carcinoma lesions tested has been found to range between 20-40%, with an average frequency of 20% (6/25 and 10/53 lesions) in two studies [39,40,70] and 35% (21/63 lesions) in the third one [71]. This frequency is lower than that found in non-SCLC, cervical carcinoma and cutaneous melanoma lesions while similar to that found in colorectal carcinoma lesions [9,72]. A comparison of the frequency of TAP1 and TAP2 downregulation observed among the different studies indicates that a similar frequency was observed for RT-PCR analysis of primary frozen tissue sections [40] and IHC staining of primary and metastatic formalin-fixed, paraffin-embedded tissue sections [70]. Therefore, it is unlikely that the conflicting results brought about by these studies reflect differences in the type of lesions analyzed, i.e. primary lesions [40,41] or primary and metastatic lesions [70] and in the methodology used to detect TAP expression. It is more likely that these conflicting results reflect the use of different substrates in the IHC staining, i.e. frozen tissue sections [71] vs formalin-fixed, paraffin-embedded tissue sections [70]. However, it cannot be excluded that these differences may also reflect differences in the characteristics of the anti-TAP1 and TAP2 xenoantibodies used for IHC staining, the criteria to classify TAP1 and TAP2 expression and/or the characteristics of the patient populations investigated.

ii. Loss of one HLA haplotype

Loss of the gene products of one of the two HLA-A, B, and C loci (one haplotype), appears to be frequently caused by loss of segments of the short arm of chromosome 6 (6p) where HLA class I genes reside [73], however in some instances it can be caused by the loss of specific transcription factors that specifically bind to HLA-A or HLA-B promoters [74]. LOH at chromosome 6 appears to represent the most frequent mechanism contributing to selective HLA haplotype loss in tumors [75]. This finding may reflect the frequent genetic recombination events at the human *MHC* located at chromosome 6p21.3, which carries the highest density of genes among all gene loci in human chromosomes [76]. Loss of one HLA class I haplotype is often identified by HLA class I genotyping and LOH analysis of chromosome 6p. To date, only one study has investigated LOH at chromosome 6p in 99 surgically removed breast carcinoma lesions and found it to be present in 25% of the lesions analyzed [46]. For comparison, this frequency is higher than that found in laryngeal and colorectal carcinoma and cutaneous melanoma lesions, while lower than that found in HNSCC, cervical carcinoma and uveal melanoma lesions [9]. It should be noted that the influence of technical and LOH classification differences among the reported studies makes it difficult to determine if these differences in LOH frequencies are cancer specific (e.g. carcinoma vs. sarcoma). Furthermore, the number of lesions analyzed to date is too low to draw conclusions regarding the frequency of LOH at chromosome 6p in breast carcinoma.

iii. Selective HLA class I antigen loss and/or downregulation

The frequency of selective HLA class I antigen loss and/or downregulation in breast carcinoma lesions averages around 20%, among most of the reported studies [25,26,29,33,71], although frequencies have been reported as high as 75% (3/4 lesions) [28] and as low as 2% (2/94 lesions) [25,26]. It is expected that the frequency of this phenotype in breast carcinoma lesions is higher than that described in the literature, since the expression of only a limited number of HLA class I allospecificities (e.g. A2, A3, A23, A24, A28, A30, A31, B5, B7, B8, B12, B17, B18 B51, Bw4 and Bw6) has been assessed in breast carcinoma lesions due to the lack of appropriate probes. The frequency of selective HLA class I antigen loss and/or downregulation in breast carcinoma lesions is higher than that found in cervical carcinoma, prostate carcinoma and cutaneous melanoma lesions and similar to that found in HNSCC, lung, renal cell and colon carcinoma lesions [Fig. 3] [9,77]. However, the number of lesions analyzed is far too low to draw definitive conclusions regarding the frequency of selective HLA class I allospecificity loss and/or downregulation in breast carcinoma lesions given the high degree of HLA antigen polymorphism.

Selective loss and/or downregulation can be caused by loss of the gene(s) which encode the heavy chain of the lost HLA class I allele(s) [78], mutations which inhibit its (their) transcription or translation [78] or alterations in the transcription factors for genes encoding HLA class I heavy chains [79,80]. In the latter case, the role of this mechanism in selective HLA class I antigen loss and/or downregulation in breast carcinoma cells has yet to be investigated. Studies with other types of tumors have shown that mutations found in HLA class I heavy chains can range from large deletions to single base deletions or substitutions and appear to occur randomly [81-85]. As in the case of the $\beta_2 m$ gene, no mutations have been reported for HLA class I heavy chains in breast carcinoma cells. However, it should be noted that, to the best of our knowledge, no study has extensively examined the HLA class I antigen heavy chain gene sequences in surgically removed breast carcinoma cells. Alternative mechanisms underlying selective HLA class I antigen loss include yet to be defined post-transcriptional mechanisms and/or genetic alterations at the chromosome 6p21.3 locus, which have been shown to result in partial HLA class I antigen loss in both cervical and prostate carcinoma cells [86,87]. More recently, a genomic region located on chromosome 1p35-36.1 has been implicated in HLA class I antigen expression and is known as the putative modifier of methylation for HLA class I genes (MEMO-1) [88,89]. Analysis of MEMO-1 LOH in 99 surgically removed breast carcinoma lesions found this phenotype to be present in 28 of the lesions (29%) [46]. The potential role of MEMO-1 allelic loss in HLA class I abnormalities in breast cancer cells is supported by the loss of expression of HLA-C antigens, and gene products of other HLA class I loci, in neuroblastoma cell lines with deletions within this region [88,89].

III. Non-classical HLA class I antigen, MICA/B, and ULBP expression by breast carcinoma cells

As discussed above, classical HLA class I antigen abnormalities have been frequently found in breast carcinoma cells. Therefore, on the basis of the missing self-hypothesis [90] one would expect that classical HLA class I antigen loss would increase the sensitivity of malignant mammary cells to NK cell killing and that tumor growth would be controlled by NK cells. Although there is *in vitro* evidence that HLA class I antigen loss or downregulation by breast carcinoma cells is associated with their increased susceptibility to NK cell-mediated lysis [91], to date there is no *in vivo* evidence of a role of NK cells in growth control of tumors with classical HLA class I antigen abnormalities in patients with breast carcinoma. These unexpected findings have stimulated interest in determining the reasons for the lack of breast carcinoma growth control by NK cells *in vivo*.

NK cell function is the result of a fine-tuning between opposite signals by inhibitory and/or activating receptors [92,93], which are generated by specific target cell ligand-NK cell receptor interactions. To date, there is evidence that the non-classical HLA class I antigens HLA-E, F, G may serve as inhibitory NK cell ligands, while the phylogenetically distant HLA class I MHC class I related chain A and B (MICA and MICB) and the UL16-binding protein 1, 2 and 3 (ULBP1, ULBP2 and ULBP3) may act as activating NK cell ligands. Furthermore, the level of NK cell-mediated cytotoxicity strictly correlates with the expression and the surface density of MICA and ULBP antigen on tumor cells of different histotypes [94]. These findings have stimulated interest in characterizing breast carcinoma cells for HLA-E, F, G antigens as well as for MIC and ULBP antigen expression, since the expression pattern of these antigens may provide a molecular mechanism for the resistance of breast carcinoma cells with classical

HLA class I downregulation to NK cell recognition and destruction. Nevertheless the available information is still limited, since the field is still in an early stage and progress in this area is hindered by the lack and/or limited availability of reagents suitable for detection of non-classical HLA class I antigens and NK cell ligands. In spite of these limitations we have reviewed the available information and summarized the results of our preliminary studies, since this is an intriguing area of research and has potential clinical implications for the rational design of immunotherapeutic strategies which take into account the antigenic profile of breast carcinoma cells.

i. Non-classical HLA class I antigen expression

To the best of our knowledge only one study has investigated non-classical HLA class I antigen expression on two breast carcinoma cell lines. Analysis of two breast carcinoma cell lines for HLA-E expression did not detect any significant amount of HLA-E expression [95]. Recently, in preliminary studies we have investigated HLA-E and HLA-F expression on 8 breast carcinoma cell lines and found them to be expressed in 50% (4 of 8) and 25% (2 of 8), respectively [Chang *et al.* unpublished]. Upon IFN-γ stimulation, HLA-E and HLA-F expression were induced or enhanced on 7 of 8 cell lines analyzed. No HLA-G expression was detected in any of the cell lines tested. In breast carcinoma lesions, only the frequency of HLA-G expression has been investigated and the results described in the literature are conflicting. Both Pangault *et al.* and Palmisano *et al.* did not detect HLA-G expression in 35 surgically removed breast carcinoma lesions [96,97]. On the other hand, Lefebvre *et al.* and Singer *et al.* reported HLA-G protein expression in 38% (14 of 36) and 25% (22 of 88) surgically removed breast carcinoma lesions, respectively [98,99]. In the study by Singer *et al.*, significantly higher levels of soluble HLA-G (sHLA-G) were detected in breast carcinoma ascites specimens as compared to benign ascitic fluid samples, suggesting that sHLA-G may represent a useful ascitic-fluid, tumor marker in patients with breast carcinoma [99]. In addition, given the ability of serum HLA-G antigens to induce apoptosis of T cells [100,101], one wonders whether soluble HLA-G antigens may have a negative impact on a patient's immune response to his own tumor and eventually on the clinical course of the disease.

ii. MICA/B and ULBP ligand expression

Only we have investigated the expression of NK activating ligands on a panel of breast carcinoma cell lines. In preliminary studies, we have found MICA, ULBP1 and ULBP3 on 50%, 75% and 75%, respectively, of the eight cell lines tested, but we have not detected MICB and ULBP2 on any of the cell lines examined [Chang *et al* unpublished]. Interestingly, the frequency of NK cell activating ligand expression appears to be independent of classical HLA class I antigen expression. With regard to NK cell activating ligand expression in breast carcinoma lesions, to the best of our knowledge, only MICA expression has been investigated in a small number of surgically removed breast carcinoma lesions and found to be expressed in 7 of the 20 lesions analyzed [102].

IV. Functional relevance and potential clinical significance of in HLA class I antigen and NK cell activating ligand abnormalities in breast carcinoma cells.

The functional and clinical relevance of HLA class I antigen abnormalities in breast carcinoma cells has been investigated only for classical HLA class I antigens. This topic has not been investigated for non-classical HLA class I antigens, although we believe this to be a fruitful area of study in view of the potential ability of non-classical HLA class I antigens to affect interactions of malignant cells with the host's immune system. In general, the frequency of classical HLA class I antigen defects is associated with disease progression, since it is higher in metastatic than in primary and premalignant lesions [9]. These findings parallel similar results in primary laryngeal and hypopharyngeal carcinoma, small cell lung carcinoma (SCLC), squamous cell carcinoma of the lung, cervical carcinoma, renal cell carcinoma, colon carcinoma and cutaneous melanoma and suggest that classical HLA class I antigen abnormalities may play a role in tumor cell escape from TA-specific CTL lysis and allow malignant epithelial cells to progress to advanced tumor grades [9,103-106]. However, analysis of approximately 900 lesions has not provided any clear indication as to whether classical HLA class I antigen abnormalities are associated with histological differentiation, abnormal DNA content and/or tumor grading [15,22,23,25,26,29,35,36,37,38]. Furthermore, there are conflicting reports regarding the association between classical HLA class I antigen abnormalities in breast carcinoma lesions and patient survival. Of the studies performed, Wintzer *et al.*, Vitale *et al.* and Gudmundsdottir *et al.* (306 total patients) [24,37,38] have not demonstrated an association between classical HLA class I antigen abnormalities and patient survival, while Concha *et al.* and Zia *et al.* (124 total patients) [26,41] have demonstrated such an association. The lack of association between classical HLA class I antigen abnormalities and patient survival as found by Wintzer *et al.*, Vitale *et al.* and Gudmundsdottir *et al.* is unlikely to be attributed to a lack of a T cell mediated immune response in the analyzed breast carcinoma patients, since the presence of TA-specific CTL has been shown in the majority of patients with breast carcinoma [107-111] and CTL established from these patients have been shown to display significant levels of cytolytic activity against known breast cancer antigens such as MUC-1 [110] and HER-2/neu [108,109]. Moreover, it is noteworthy that detection of HLA class I antigens in tissues with IHC assays does not exclude that they are non-functional or malfunctional because of mutations and/or changes in their conformation. It is more likely that the lack of association of classical HLA class I antigen abnormalities with the histological type, degree of tumor cell differentiation, clinical stage, disease free interval and/or survival in breast carcinoma may be attributed to differences in the characteristics of the patient population, the methods of analysis, the system used to score classical HLA class I antigen expression and/or the confounding effect of these variables.

The low frequency of NK activating ligand expression in the very small number of breast carcinoma cell lines and surgically removed breast carcinoma lesions provides a molecular mechanism for the lack of a role of NK cells in the control of breast carcinoma cell growth *in vivo*. These findings stress the need to examine a large number of breast carcinoma lesions for NK activating ligand expression and correlate these findings with the expression of classical and non-classical HLA class I antigens as well as susceptibility of breast carcinoma cells to NK cell-mediated lysis *in vitro* and with the clinical course of the disease. These studies should also investigate the potential role of serum HLA-G and MIC antigens, since both molecules in a soluble form downregulate the activity of T cells and possibly NK cells, both of which express NKG2D, the receptor for MICs [101,102]. This information will help to optimize immune based strategies for the treatment of breast carcinoma.

V. HLA class II antigen expression in breast carcinoma lesions

i. HLA class II antigens

To date, IHC staining of over 1000 surgically removed breast carcinoma lesions has shown that HLA class II antigens are often up-regulated on malignant tumors of mammary epithelial cells [14,19,23,24,26,32,112-121]. As observed in colon and cervical epithelial cells [122,123], HLA class II antigen expression does not appear to be restricted to cells which have undergone malignant transformation, since IHC analysis has also identified HLA class II antigen expression in benign mammary lesions [19,23,26,114,115]. The frequency of HLA class II antigen expression in breast carcinoma lesions has been reported to average around 30%, however frequencies have been noted to vary between 13% (10/77) [24] and 89% [113].

The differences among the reported frequencies of HLA class II antigen expression in breast carcinoma lesions are likely to result from the effect of many variables including patient population characteristics, sample size, criteria utilized to score HLA class II antigen expression and characteristics of anti-HLA class II mAb used. The latter include mAb, which recognize determinants shared by HLA-DR, DQ and DP antigens, e.g. mAb Q5/13, the anti-HLA-DR antigen mAb including FMC14, GRB1, ISCR3, L243, L227, M704, M775, Q2/70, Q5/6 and TAL-1B5, and the anti-HLA-DQ antigen mAb used include TU22 and SK10. All studies of HLA-DP antigen expression have utilized the anti-HLA-DP antigen mAb B7/21. Another source of variability may be attributed to the histological type of tumors analyzed, since the frequency of HLA class II antigen expression has been found to be higher in medullary breast carcinoma than in atypical medullary or invasive ductal breast carcinomas [119,120]. However, it is noteworthy that the frequency of HLA class II antigen expression does not vary among all histological types of breast carcinoma, since no significant difference in the frequency of HLA class II antigen expression has been observed among ductal, lobular and mucinous histotypes [23]. All the studies performed have investigated HLA-DR antigen expression [14,19,23,24,26,32,112-121,124,125], while only five have examined HLA-DP and HLA-DQ antigen expression. In all five studies, irrespective of the frequency of HLA class II antigen detection in the breast carcinoma lesions analyzed, HLA-DR antigens have been found to be more frequently expressed than HLA-DQ and DP antigens [23,32,116,124,125]. The differential expression of the gene products of HLA class II loci is not unique of breast carcinoma cells, since it has been described also in colon carcinoma [126] and cutaneous melanoma cells [127].

Only limited information is available about HLA class II allospecificity expression in breast carcinoma lesions. Most studies have examined HLA-DR, -DP and -DQ antigen expression, utilizing locus specific mAb due to the lack of availability of allospecific mAb which recognize determinants expressed in formalin-fixed and paraffin-embedded tissue sections. Furthermore, the determinants recognized by the allospecific mAb that have been utilized are influenced not only by allelic polymorphisms in the HLA class II antigen peptide binding site, but also by the array of bound peptides. Therefore, the reported frequencies of HLA class II allospecificity expression should be interpreted with caution. In this regard, using a panel of non-conformational dependent HLA-DR allospecific mAb, two recent studies demonstrated selective HLA-DR allospecificity expression on IFN-γ-treated breast carcinoma cell lines and 6/15 breast carcinoma lesions [121,128].

ii. HLA class II co-chaperone Ii and non-classical HLA class II antigen expression

HLA class II co-chaperone Ii has been found to be more frequently expressed than HLA class II antigens in breast carcinoma lesions [23,116,121]. While the significance of Ii expression has not yet been evaluated in breast cancer, high Ii expression in colorectal [129] and gastric carcinoma [130] lesions is associated with a poor prognosis. If not fortuitous, this association may be due to the prevention of TA derived peptide presentation to CD4+ T cells for recognition, since *in vitro* studies have shown that HLA class II-transfected tumor cells are unable to present TA derived peptides to CD4+ T cells in the presence of Ii [131-132]. In regards to the non-classical HLA class II antigen, HLA-DM, no published studies have examined its expression in breast carcinoma lesions. However, preliminary investigations have found HLA-DM to be infrequently expressed and at very low levels in comparison to Ii expression in breast carcinoma lesions [Oldford & Drover, unpublished].

VI. Potential mechanisms underlying HLA class II antigen expression in breast carcinoma cells

Information regarding the molecular mechanism(s) underlying HLA class II antigen up-regulation on breast carcinoma cells is presently scanty. In contrast to the expression of HLA class I antigens on most nucleated cells [16], HLA class II (HLA-DR, -DP and –DQ) antigen expression is more tightly regulated [133]. Conventional antigen presenting cells (APC), such as B-cells, macrophages and dendritic cells, as well as thymic epithelial cells, constitutively express HLA class II antigens and these levels can be augmented in response to IFN-γ. Both constitutive and IFN-γ inducible HLA class II antigen expression are controlled by the class II transactivator (CIITA), with constitutive and inducible HLA class II antigen expression regulated by CIITA promoters III and IV, respectively [134]. In the following sections we will discuss the potential molecular mechanisms underlying HLA class II antigen expression in breast carcinoma lesions.

i. Cytokine and/or hormonal modulation

HLA class II antigen up-regulation on breast cancer cells may be attributable to the cytokine milieu within the tumor microenvironment, largely influenced by cytokine producing infiltrating T cells [135]. Factors such as interleukin-1α (IL-1α) and estradiol, [136], tumor necrosis factor-α (TNF-α) [59] and IL-4 [137] have been shown to modulate HLA class II antigen expression. However, it is generally assumed that the mechanism by which HLA class II antigens are up-regulated on breast carcinoma cells is via the IFN-γ inducible pathway, since constitutive HLA class II antigen expression is associated with constitutive CIITA transcription in melanoma cells [138]. Indeed IFN-γ can induce HLA class II antigen expression on many cell types including breast carcinoma cell lines [59,128,131,132,139-143]. However, to the best our knowledge the frequency of CIITA expression in breast carcinoma lesions has yet to be investigated. Therefore, the role of IFN-γ in the up-regulation of HLA class II antigens in breast carcinoma lesions still remains unclear.

It is noteworthy that no clear relationship has been established between HLA class II antigen expression and T cell infiltration of breast carcinoma lesions [19,20,22,26,121]. An alternative and distinct mechanism underlying HLA class II antigen up-regulation on breast carcinoma cells is represented by molecular changes associated with malignant transformation of mammary epithelial cells and/or disease progression. In this regard, the association between HLA-DR antigen and estrogen and progesterone receptor expression in breast carcinoma lesions suggests a role for hormonal control of HLA class II antigen expression [124]. Although these findings have not been confirmed [24,113,118], the potential regulation of HLA class II antigen expression by hormones is also supported by HLA class II antigen up-regulation on mammary epithelial cells in the lactating breast in response to prolactin [112,144,145].

ii. Promoter polymorphisms

Differential HLA class II antigen isotype expression (DR, DQ and DP) and DRB alleles by tumor cells may be due to promoter polymorphisms [146-150]. Differences in DRB promoter transcriptional activity and levels of DRB mRNA suggest that differential DRB gene expression is regulated at both transcriptional and post-transcriptional levels [151,152]. Thus, factors produced during tumorigenesis may contribute to both transcriptional and post-transcriptional control of HLA-DR allospecificity expression on tumor cells.

iii. Defective HLA class II antigen expression

As stated before HLA class II antigen up-regulation is not detectable in about 50% of breast carcinoma lesions. It is likely that this finding reflects multiple molecular mechanisms. Among them, one is suggested by the combined lack of constitutive HLA class II antigen expression and resistance to IFN-γ mediated induction of HLA class II antigen expression which has been documented in several breast carcinoma cell lines, although the *in vivo* relevance of these findings remains still to be determined. Furthermore, LOH of chromosome 6p is likely to play a role in the lack of HLA class II antigen expression, since this mechanism causes HLA haplotype loss in a high percentage of tumors [75] and mutations in the remaining copy of chromosome 6 would then result in lack of HLA antigen expression. The role of this combined mechanism is supported by the increased frequency of HLA class I antigen loss in HLA class II antigen negative tumors [23,121]. Defects in transcriptional regulation may also be responsible for defective HLA class II antigen expression in breast carcinoma cell lines. In this regard, defective CIITA transcription has been noted in several breast carcinoma cell lines because of hypermethylation of its promoter IV [153,154], modification by histone deacetylation [155] or defects in the retinoblastoma tumor suppressor protein (RB) [156]. More recently, CIITA transcription was found to be reduced by over expression of the oncogenes, L-myc and N-myc, as well as of the human achaete-scute homolog in a SCLC and neuroblastoma cell line [157].

Lastly, defects in posttranscriptional regulation are suggested to play a role in the lack of HLA class II antigen expression by the transcription of HLA class II gene(s) in breast carcinoma cell lines which do not express HLA class II proteins [143].

VII. Functional and clinical significance of HLA class II antigen expression in breast carcinoma lesions

The functional significance of HLA class II antigen up-regulation on breast carcinoma cells is controversial, since unlike conventional APC, HLA class II antigen-positive tumor cells lack co-stimulatory molecules, which are required to initiate an optimal CD4+ T-cell response. Nevertheless, there is substantial experimental evidence to support a role for tumor cell HLA class II antigen expression in anti-tumor immunity [1,132,133,158]. In this regard, convincing evidence indicates that HLA class II antigen positive breast carcinoma cells have the ability to generate and present the relevant TA derived peptides as well as to activate effector CD4+T-cells in a HLA class II antigen restricted manner both in mice [159] and in humans [160-162]. However, whether this TA-specific CD4+ T cell response actually plays a role in controlling tumor growth in breast carcinoma patients remains unclear.

HLA class II antigen expression in breast carcinoma lesions has not been found to be associated with tumor stage [23], mitotic index [117,118] or lymph node metastases [32,117]. Furthermore, there is conflicting evidence regarding the association between HLA class II antigen expression and differentiation state of the tumor [22,23,26,118,124]. However, a recent study suggests that these discrepancies may in fact be related to differences in the amount T cell tumor infiltration, since HLA-DR antigen expression was found to be associated with well-differentiated tumors that lack a T cell infiltrate [121]. Therefore, HLA class II antigen up-regulation in breast carcinoma lesions may result from the presence of T cell derived modulatory cytokines or molecular changes that occur during malignant transformation because of the genetic instability of tumor cells.

Several explanations can be envisioned for the conflicting information about the prognostic significance of HLA class II antigen expression in breast carcinoma lesions. First, detection of HLA class II antigens in tissues with IHC assays does not exclude that they are non-functional or malfunctional because of mutations and/or changes in their conformation. Second, associations (or lack of) are likely to be influenced by the complexity of breast carcinoma, which is not a single disease entity, and is characterized by many different interacting factors. For example, the most common breast carcinoma, invasive ductal, is more likely to be poorly differentiated, hormone receptor negative and to occur in younger women, whereas the next most common type, invasive lobular breast carcinoma, is better differentiated, likely to be hormone receptor positive and to occur in older women. Thus, the interplay of these factors combined with hormones and cytokines in the tumor microenvironment is likely to influence up-regulation of HLA class II antigen expression by tumor cells. Furthermore the analysis of the clinical significance of HLA class II antigen expression in breast carcinoma lesions has, in general, not taken into account the expression of other types of histocompatibility antigens, as well as other molecules which are likely to greatly affect the interactions of breast carcinoma cells with host's immune system. Therefore, comprehensive studies, which take into account the interplay of immunologically relevant markers on breast carcinoma cells and can be subjected to rigorous multivariate statistical analysis, are needed to assess the functional role of HLA class II antigen expression in the clinical course of breast carcinoma and its prognostic significance.

VIII. Conclusion

It is clear that both defects in classical HLA class I antigen expression and up-regulation of HLA class II antigens occur in mammary epithelial cells which have undergone malignant transformation. However, only scanty information is available regarding the molecular mechanisms underlying these changes as well as their clinical significance. Furthermore no information is available about the functional characteristics of HLA class I and class II antigens expressed by breast carcinoma cells, specifically about their ability to present TA derived peptides to T cells. The lack of this information reflects the fact that probes to measure HLA class I and class II antigen-TA derived peptide complexes on tumor cells are at present not available. In view of the crucial role played by these complexes in the recognition of breast carcinoma cells by host's T cells, it is inherent that these probes are developed especially since the technology to generate them is presently available. Another deficiency that has emerged from the review of the literature is the paucity of information regarding the frequency of non-classical HLA class I antigen and NK cell activating ligand expression in breast carcinoma lesions. It is hoped that this information will become available in the near future not only because of the critical role of these molecules in the interactions of breast carcinoma cells with immune effector cells, but also because of the potential usefulness of these molecules to develop novel immunotherapeutic strategies for the treatment of breast carcinoma.

One might ask whether the study of HLA antigen expression has relevance in breast carcinoma research. It is our belief that these studies are important and timely since they can provide insights into the host immune system's role in the control of breast carcinoma cell growth and can help to optimize the implementation of immunotherapy of breast carcinoma. In this regard the identification of HLA antigen abnormalities in breast carcinoma lesions is compatible with the possibility that the host mounts an immune response against breast carcinoma cells and the resulting immune selective pressure lead to the overgrowth of tumor cells which have developed escape mechanisms. In view of the potential role of a patient's immune response in the generation of breast carcinoma lesions with HLA defects, our understanding of the dynamic changes in the antigenic phenotypes of breast carcinoma lesions will benefit from studies which correlate HLA antigen defects with the type of immune response present in a patient. From a clinical viewpoint these findings emphasize the need to characterize and monitor HLA antigen expression in breast carcinoma lesions in order to optimize the selections of patients who are likely to benefit from immiunotherapy. Furthermore, the frequent antigenic changes convincingly documented in surgically removed breast carcinoma lesions stress the importance of modality treatments which utilize multiple and distinct arms of the immune system in combination with non-immunologically based strategies for successful therapy of breast carcinoma.

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Figure 1. Interaction of classical and non-classical HLA class I antigen, and natural killer (NK) cell activating ligands MICA/B and ULBP with T cells and NK cells. (A) Classical HLA class I antigens play a major role in the interactions between target cells and (*1*) activation of peptide-specific CTL through TCR; (*2*) inhibition of T cell subpopulations through inhibitory receptors KIR or (*3*) T cell subpopulations through CD94/NKG2A; (*4*) inhibition of NK cell-mediated killing through KIR. **(B)** Non-classical HLA class I antigens play a major role in the interactions between target cells and inhibition of NK cell-mediatied killing through (*5*) HLA-E antigen-CD94/NKG2A interactions or (*6*) HLA-G antigen-KIR interactions. NK cell activating ligands MICA/B and ULBP play a major role in the interactions between target cells and activation of NK cells through (*7*) NKG2D. **(C) Role of HLA class II antigens in the generation of an antitumor immune response.** HLA class II antigens play a major role in the interaction between target cells and (*1*) activation of macrophage through release of Th1 cytokines; (*2*) activation of eosinophils through release of Th2 cytokines, and (*3*) activation of CTL through release of Th1 cytokines.

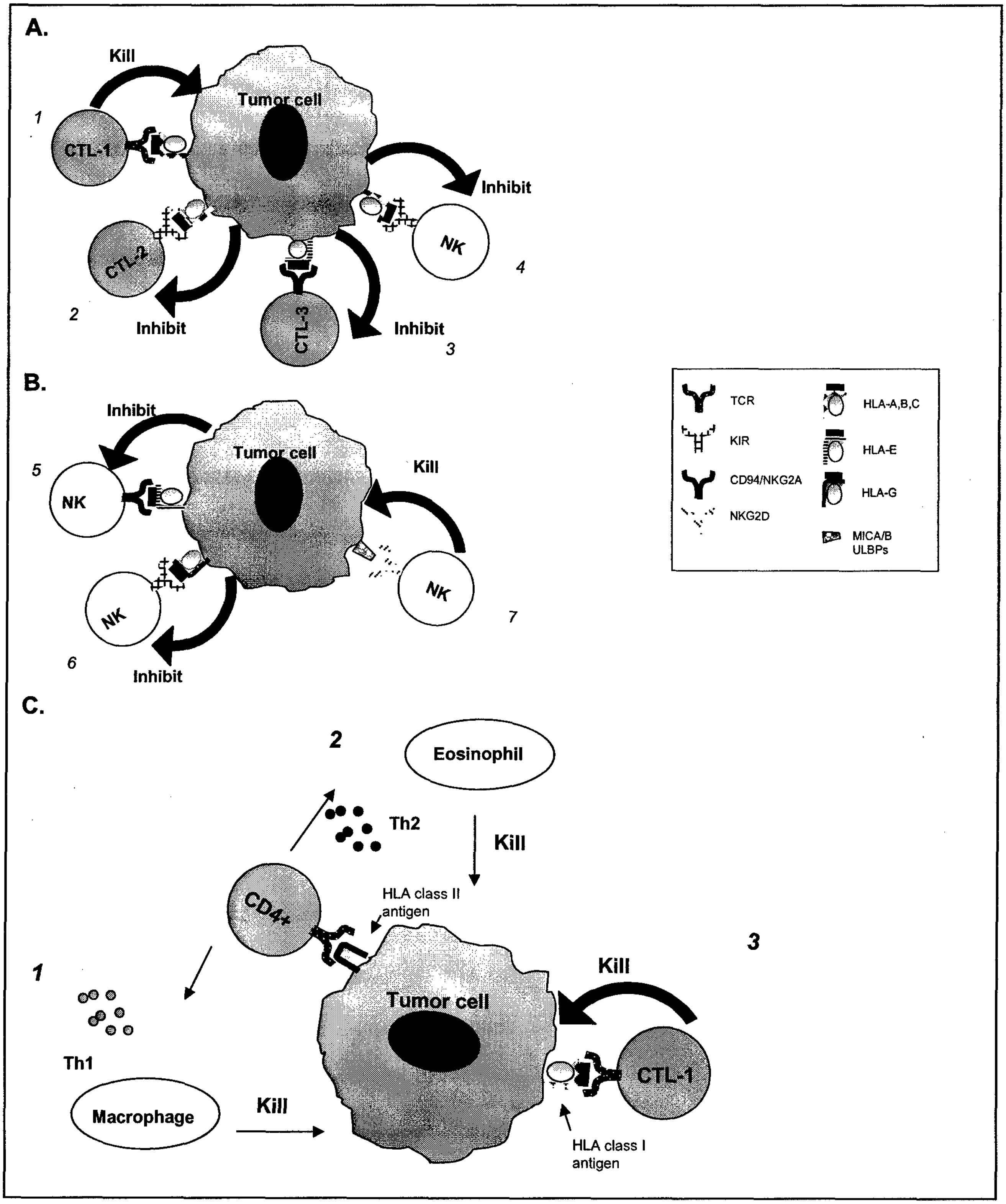
Figure 2. Defective HLA class I phenotypes identified in breast carcinoma lesions. The phenotypes identified in tumor cells include: (A) total loss and/or downregulation of the gene products of the HLA-A, B and C loci; (B) selective loss and/or downregulation of the gene products of one HLA class I locus; (C) selective loss of one HLA class I allospecificity, or (D) total loss of all HLA class I antigens encoded in one haplotype.

Figure 3. Frequency of HLA class I antigen abnormalities in malignant lesions of different embryological origin. The most common types of solid tumors for which more than 300 lesions have been analyzed for HLA class I antigen expression are shown. (■) Indicates total HLA class I antigen loss and/or downregulation; (▒) indicates selective HLA class I allospecificity loss;

Figure 4. Generation and interaction of HLA class I antigen-peptide complexes with T cells and NK cells. Intracellular protein antigens, which are mostly endogenous, are marked for ubiquitination within the cytosol and subsequently degraded into peptides by the proteasome. Peptides are then transported into the ER through TAP. Nascent, HLA class I antigen heavy chains are synthesized in the ER and associate with the chaperone immunoglobulin heavy chain binding protein (BiP), a universal ER chaperone involved in the translation and insertion of proteins into the ER. Following insertion into the ER, the HLA class I heavy chain associates with the chaperone calnexin and the thiol-dependant reductase ERp57. Calnexin dissociation is followed by HLA class I heavy chain association with β_2m, tapasin and the chaperone calreticulin. Calnexin, calreticulin and ERp57 play a role in folding of the HLA class I heavy chain. Tapasin brings the HLA class I heavy chain, β_2m, chaperone complex into association with TAP and plays a role in both quantitative and qualitative peptide selection. The trimeric HLA class I-β_2m-peptide complex is then transported to the plasma membrane where it plays a major role in the interactions between target cells and activation of peptide-specific CTL through TCR.

Figure 5. Quantitative analysis of antigen processing machinery component expression in human breast carcinoma cell line BT20. Cultured human breast carcinoma cell line BT20 was analyzed for proteasome subunits delta, MB1 and Z, immunoproteasome subunits LMP2, LMP7 and LMP10, and TAP1, calnexin, calreticulin, ERp57 and tapasin, expression by intracytoplasmic flow cytometry with anti-delta mAb SY-5, anti-MB1 mAb SJJ-3, anti-Z mAb NB-1, anti-LMP2 mAb SY-1, anti-LMP7 mAb SY-3, anti-LMP10 mAb TO-6, anti-TAP1 mAb TO-1, anti-TAP2 mAb NOB-2, anti-calnexin mAb TO-5, anti-calreticulin mAb TO-11, anti-ERp57 mAb TO-2, and anti-tapasin mAb TO-3, both prior to) and followi( ) *in vitro* ινχυβατιον οφ χελλσ ωιτη ΙΦΝ–γ.

Figure 1



A.
Kill
Tumor cell
1
CTL-1
Inhibit
NK
4
CTL-2
2
Inhibit
CTL-3
Inhibit
3
B.
Inhibit
Tumor cell
5
NK
Kill
NK
7
NK
6
Inhibit
TCR
HLA-A,B,C
KIR
HLA-E
CD94/NKG2A
HLA-G
NKG2D
MICA/B
ULBPs
C.
2
Eosinophil
Th2
Kill
CD4+
HLA class II
antigen
3
1
Kill
Tumor cell
Th1
CTL-1
Macrophage
Kill
HLA class I
antigen

Figure 2

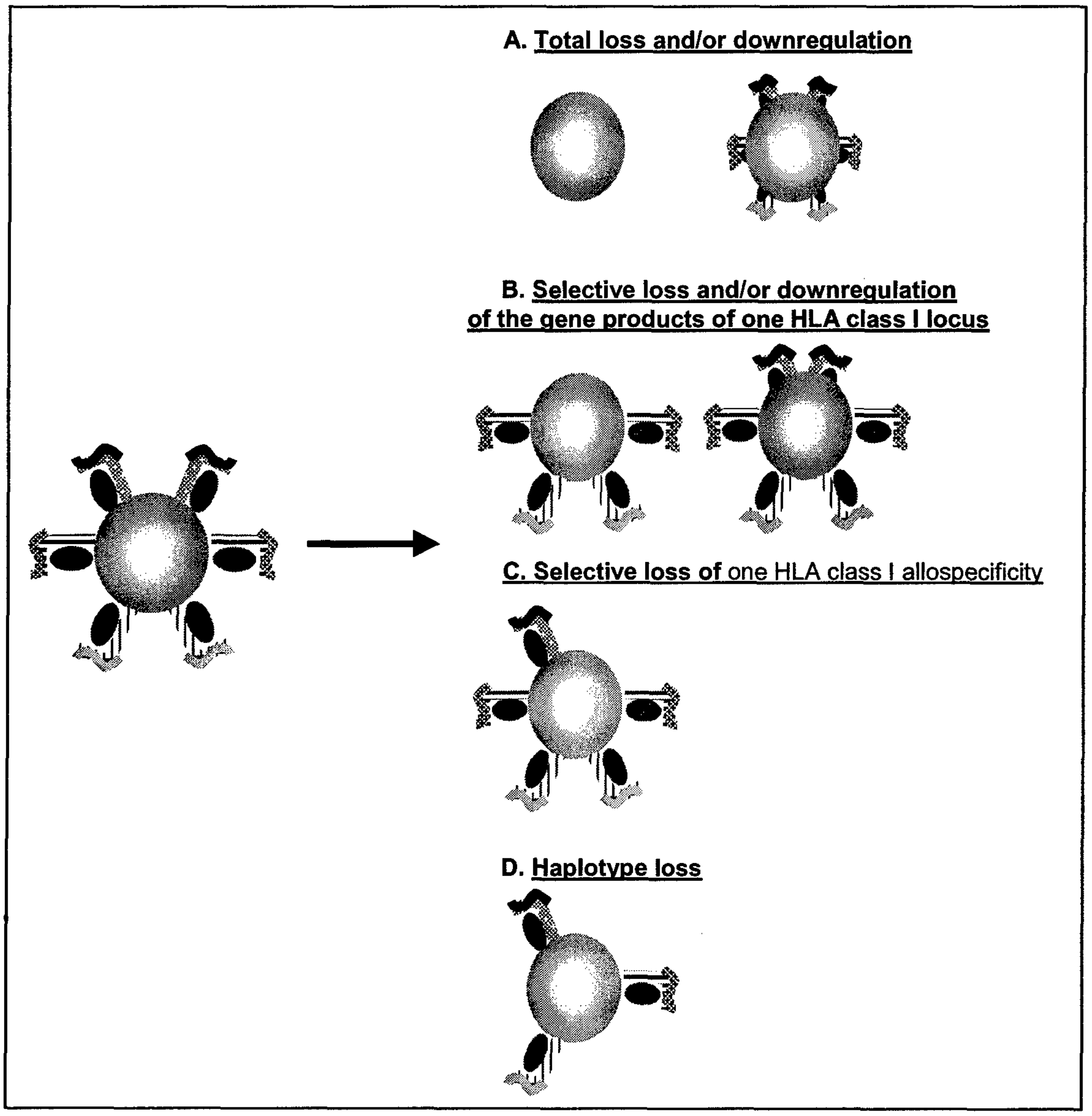
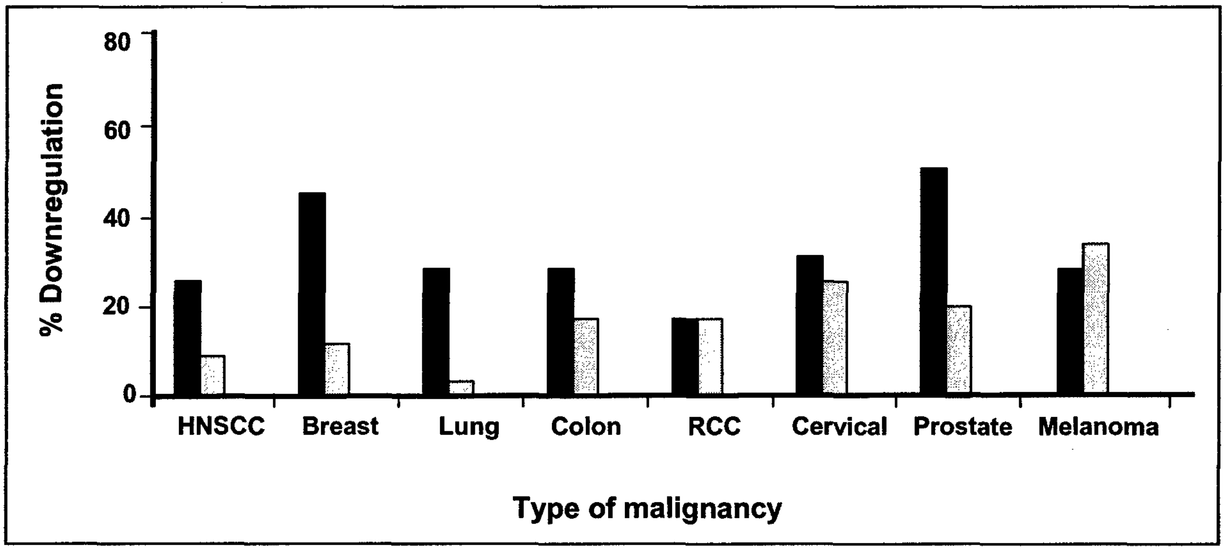

A. Total loss and/or downregulation
B. Selective loss and/or downregulation
of the gene products of one HLA class I locus
C. Selective loss of one HLA class I allospecificity
D. Haplotype loss

Figure 3



80
60
40
20
0
% Downregulation
HNSCC
Breast
Lung
Colon
RCC
Cervical
Prostate
Melanoma
Type of malignancy

Figure 4

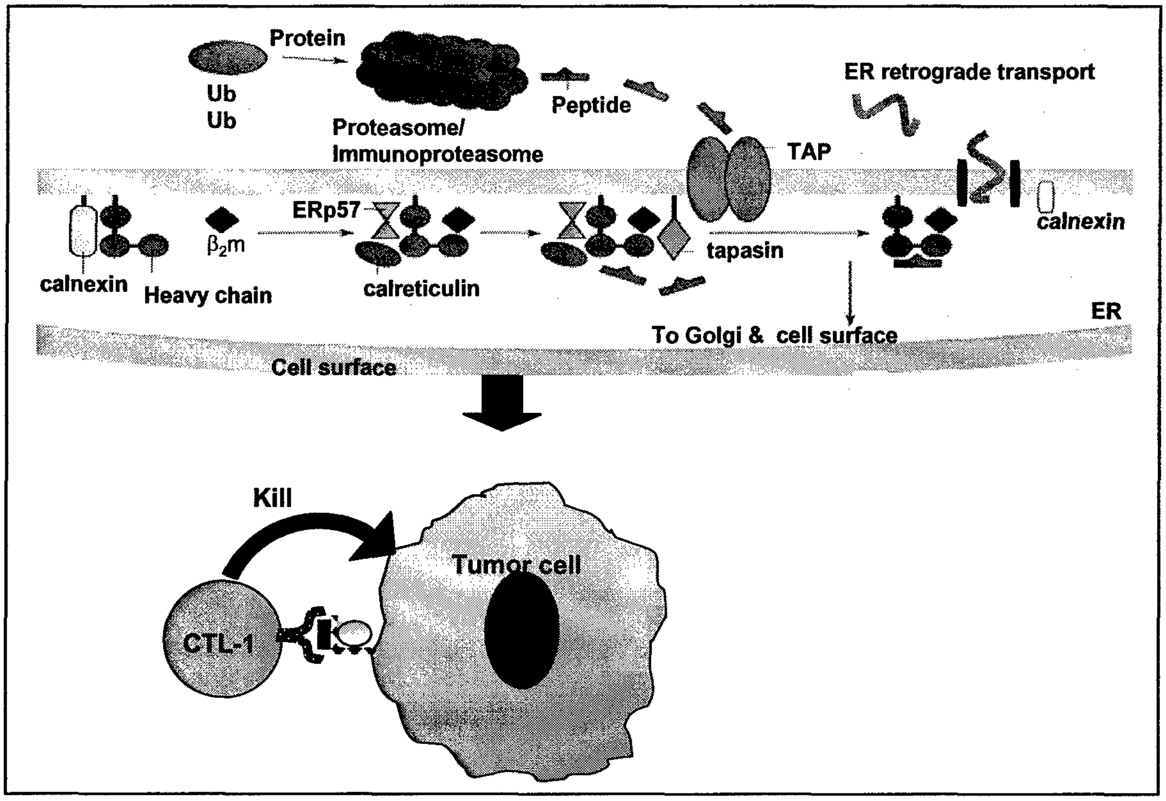
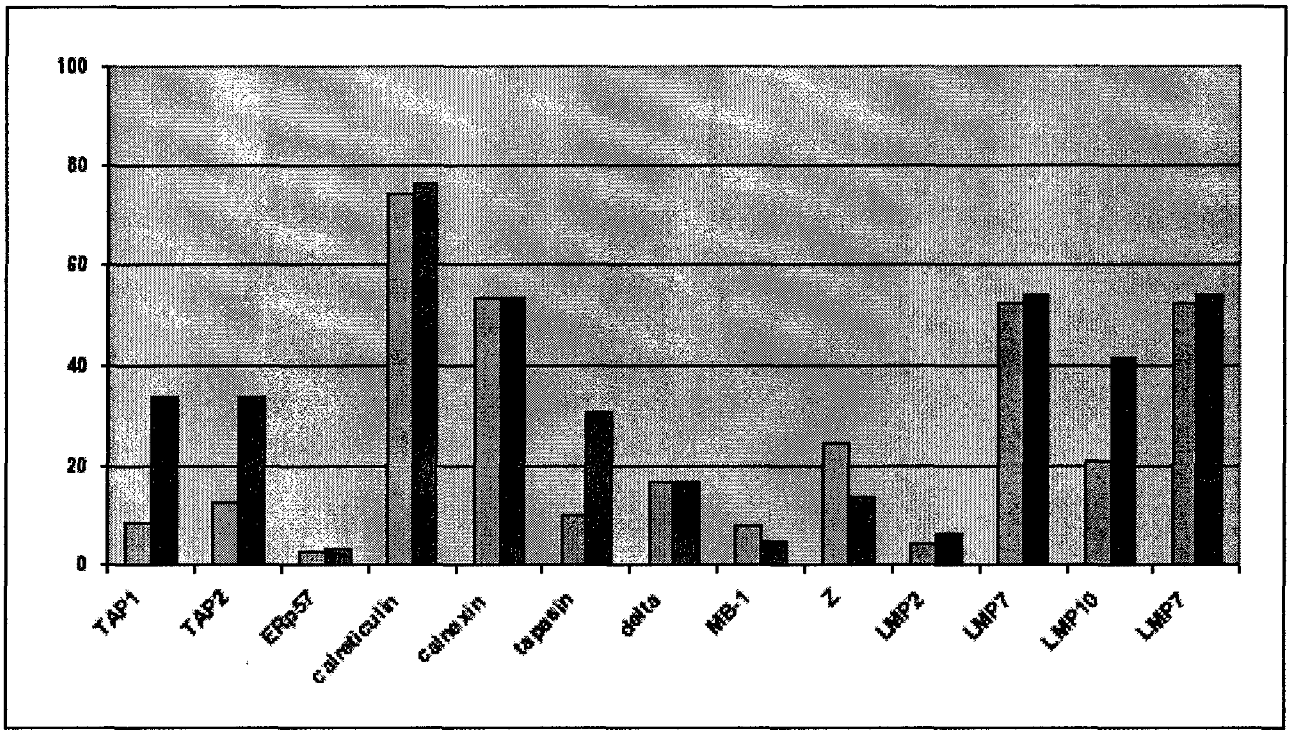

Protein
Ub
Ub
Proteasome/
Immunoproteasome
Peptide
ER retrograde transport
TAP
ERp57
calnexin
β_2m
Heavy chain
calreticulin
tapasin
calnexin
ER
To Golgi & cell surface
Cell surface
Kill
Tumor cell
CTL-1

Figure 5


100
80
60
40
20
0
TAP1
TAP2
ERp57
calreticulin
calnexin
tapasin
delta
MB-1
Z
LMP2
LMP7
LMP10
LMP7